Universidade de Lisboa

Faculdade de Farmácia

Pancreatic cancer. New screening biomarkers and detection methods

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Resumo

O cancro do pâncreas posiciona-se em quarto lugar como o de maior letalidade, com mais de 90% dos doentes diagnosticados nos estadios III e IV. A taxa de sobrevivência de cinco anos é de apenas 8,5%, o que pode ser explicado pela ausência de sintomas específicos e pela falta de uma estratégia de diagnóstico padronizada, de sensibilidade e precocidade acrescida. Pelo facto de o tumor primário necessitar de pelo menos cinco anos para obter capacidade metastática, o diagnóstico *early-stage* (como forma de melhorar a qualidade de vida e sobrevivência dos pacientes com cancro pancreático) é possível. Urge assim, uma atualização baseada na especificidade e sensibilidade, na atualização de biomarcadores, facilmente implementados na estratégia laboratorial e, economicamente concretizável.

Palavras-chave: biomarcador, cancro pancreático, diagnóstico early-stage

Abstract

Pancreatic cancer ranks fourth as the most lethal with more than 90% of patients being diagnosed in stages III and IV. The five-year survival rate is only 8.5%, which can be explained by the absence of specific symptoms and by the lack of a standardized guideline for an early-stage diagnostic strategy with high sensitivity. Due to the fact that the primary tumor requires at least five years to obtain metastatic capacity, early-stage diagnosis (as a way to improve the life and survival chance of pancreatic cancer patients) is possible. However, there is an urge for an update in specificity and sensitivity and an update in biomarkers easily implemented in laboratory strategy and economically feasible.

Keywords: biomarker, pancreatic cancer, early-stage diagnosis

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1 Introduction

Cancer is one of the biggest public health problems worldwide, as it is the major cause of mortality and morbidity (Mordente *et al.*, 2015). The lifetime risk of developing cancer is of almost 40% (National Cancer Institute, 2018).

Cancer is a term defining a wide variety of pathologies that can occur in almost any part of the body. Its common properties include local invasion and distant spread.

It develops when there is an accumulation of genetic damage in cells over time, due to the failing of the normal corrective processes (figure 1). The cells acquire abnormal qualities, namely rapid cell division, immortality and invasion of surrounding tissue (World Cancer Research Fund/American Institute for Cancer Research, 2018).

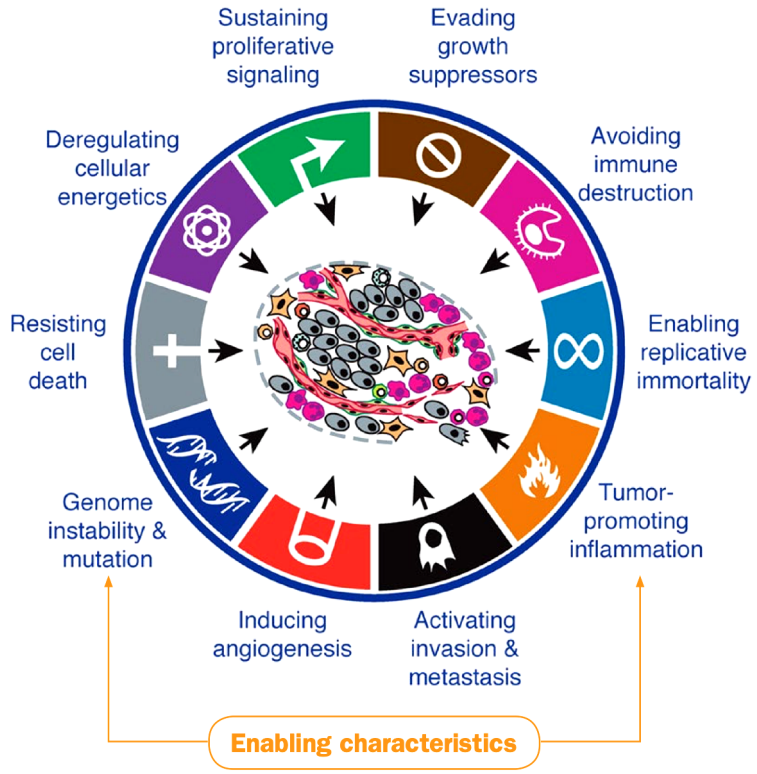


Figure 1. Hallmarks of cancer

(adapted from Hanahan *et al.*, 2011, with permission from Elsevier)

There are many tumor causing factors. Its composition, evolution, diagnosis and treatment also vary according to its subtype (La Thangue *et al.* 2011).

There is currently still an imperative need to develop a new, sensitive, specific and easy way to enable early diagnosis (Makawita *et al.*, 2010). Tumor biomarkers are a

very promising, non-invasive and quick way for early diagnosis, staging and monitoring disease progression and therapeutic response.

1.1 Pancreatic Cancer

The pancreas is a gland localized behind the stomach, containing two different parts: exocrine and endocrine. While the exocrine pancreas is responsible for the production of digestive enzymes, the endocrine pancreas produces hormones, like insulin and glucagon, being therefore included in the metabolism of glucose (World Cancer Research Fund, 2018).

Pancreatic cancer is not a single disease but a large number of neoplasms being divided into the two main pancreas functions: exocrine and endocrine.

The majority of the pancreatic cancers develop in the exocrine pancreas (about 95%) with most of them being localized in the pancreas head (World Cancer Report, 2014), namely adenocarcinoma (Vincent *et al*., 2011, Ferlay *et al*., 2012, Hidalgo *et al*., 2013). The most aggressive pancreatic neoplasm is the ductal adenocarcinoma (Hruban *et al*., 2007). Nevertheless, the early-detection of ductal adenocarcinoma improves the overall survival (Matsuno *et al*., 2004). Concerning the endocrine tumors, they make up for less than 5% of cases (Vincent *et al*., 2011, Ferlay *et al*., 2012, Hidalgo *et al*., 2013) and have a better five-year survival rate (65%) (Hruban *et al*., 2007). The most common benign pancreatic neoplasm is the serous cystadenoma (Tseng *et al*., 2005). Almost all patients with this type of tumor are cured (World Cancer Report, 2014).

Pancreatic cancer is the fourth cancer with more related deaths (Siegel *et. al* 2018). Because of the diagnosis difficulties, it is normally not diagnosed in time, having an extremely high mortality and poor prognosis (Bunger *et al*., 2011). According to Brierly *et al*. (2017), more than 90% of patients are diagnosed only at stages III and IV.

According to the National Cancer Institute (2018), the number of deaths caused by pancreatic cancer has recently outweighed the ones caused by breast cancer (table 1).

Table 1. Comparison of breast and pancreatic cancer

(adapted from National Cancer Institute, 2018)

Breast Cancer		Pancreatic cancer	
At a Glance		At a Glance	
Estimated New Cases in 2018	266,120	Estimated New Cases in 2018	55,440
% of All New Cancer Cases	15.3%	% of All New Cancer Cases	3.2%
Estimated Deaths in 2018	40,920	Estimated Deaths in 2018	44,330
% of All Cancer Deaths	6.7%	% of All Cancer Deaths	7.3%
Percent Surviving 5 Years	89.7%	Percent Surviving 5 Years	8.5%
2008-2014		2008-2014	

Due to the fact that there is a lack of standardized international guideline (Zhang *et al*., 2018a) regarding its diagnostic strategy, even for high-risk populations (Bunger *et al*., 2011), nor specific symptoms (World Cancer Research Fund, 2018), pancreatic cancer is normally diagnosed in metastatic stage (Stathis *et al*., 2010). Metastasis is the most prevailing motive of death in cancer patients (Leary *et al*., 2010). It consists in the dissemination and growth of the neoplastic cells away from the primary tumor (Yachida *et al*., 2010).

After diagnosis, very few individuals survive five years, namely 8,5% (National Cancer Institute, 2018). These poor rates have not been improving in the last 40 years (figure 2) (Zhang *et al*., 2018a).

Figure 2. Five-year survival rate

(adapted from National Cancer Institute, 2018)

Almost 80% of the diagnosed patients have spread cancer, either in regional or metastatic phase (Costello *et al*., 2012), having no opportunity to go through a curative treatment, as the surgical resection (which is the only curative option) is only possible in early stages (Yachida *et al*., 2010). According to the National Cancer Institute (2018), only 9,7% of diagnosed patients with pancreatic cancer are at local stage (figure 3). This majority of patients do not have symptoms or radiologically visible manifestations either as the disease progresses to the metastatic phase (Ghaneh *et al*., 2007, Hidalgo *et al*., 2010) making the early detection, with the current available methods, still very difficult. Nevertheless, the survival rates improve, if the pancreatic cancer is diagnosed,

and treated, at an early stage, confined to the primary site (Herreros-Villanueva, *et al*. 2016).

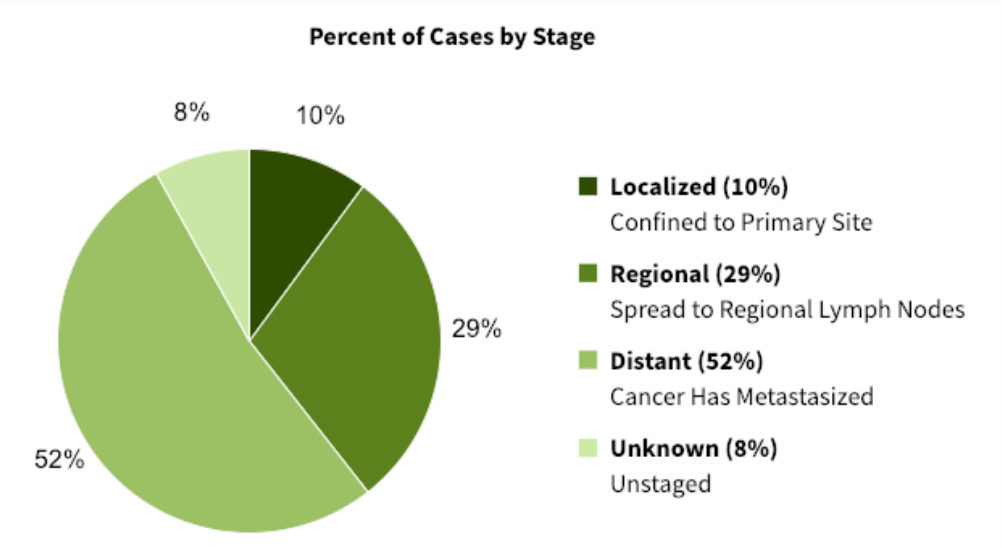


Figure 3. Percent of cases by stage of diagnosis
(adapted from National Cancer Institute, 2018)

In addition to that, cancer screening programs for other cancers, like breast, colorectal, cervical and lung cancer, have proven to reduce its mortality rates (Hakamaet *et al*., 1986, Lynge *et al*., 1989, Mandel *et al*., 1999, Sigurdsson *et al*., 1999, Tabár *et al*., 2000, Nyströmet *et al*., 2002, Atkin *et al*., 2010, Aberle *et al*., 2011, Schoenet *et al*., 2012, Hur *et al*., 2016), due to the fact that screening programs enable and increase the likelihood of an early-stage diagnosis. In early-stage, the tumor is less aggressive and still not metastatic making the chances of undergoing through curative measures majorly higher.

According to Yachida *et al*. (2010) and Poruk *et al*. (2013), in pancreatic cancer there are required at least five years, for the primary tumor to acquire the metastatic ability. During these five years the early diagnosis and prevention of deaths from pancreatic cancer could be fulfilled. Studies of Lennon *et al*. (2014), show that from the first mutation in the pancreas to the development of mutation sites, there could be a window of opportunity of detection of up to two decades. This is why, one of the most promising ways to improve the prognosis of pancreatic cancer is to develop effective early-detection strategies (Zhou *et al*., 2017).

Nevertheless, current methods are not sufficient to improve the early-diagnosis (Rulyak *et al*., 2003). The current diagnostic methods include, Computed Tomography (CT), Magnetic Resonance Imaging (MRI) and invasive endoscopic techniques

(Bunger *et al*., 2011), which are not efficient nor comfortable for the patient. Especially in comparison over plasmatic biomarkers, for which you only need a blood sample (which remain the most promising approaches to improving long-term survival (Llop *et al*., 2018).

1.2 Epidemiology

According to the World Health Organization (2019) "Epidemiology is the study of the distribution and determinants of health-related states or events (including disease), and the application of this study to the control of diseases and other health problems." It is therefore a basic science of public health that deals with incidence, distribution and possible control of diseases, among others.

Pancreatic cancer is a disease occurring almost three times more frequently in developed countries (figure 4), as is the case of Europe, Asia and Northern and South America (Ferlay *et al*., 2010).

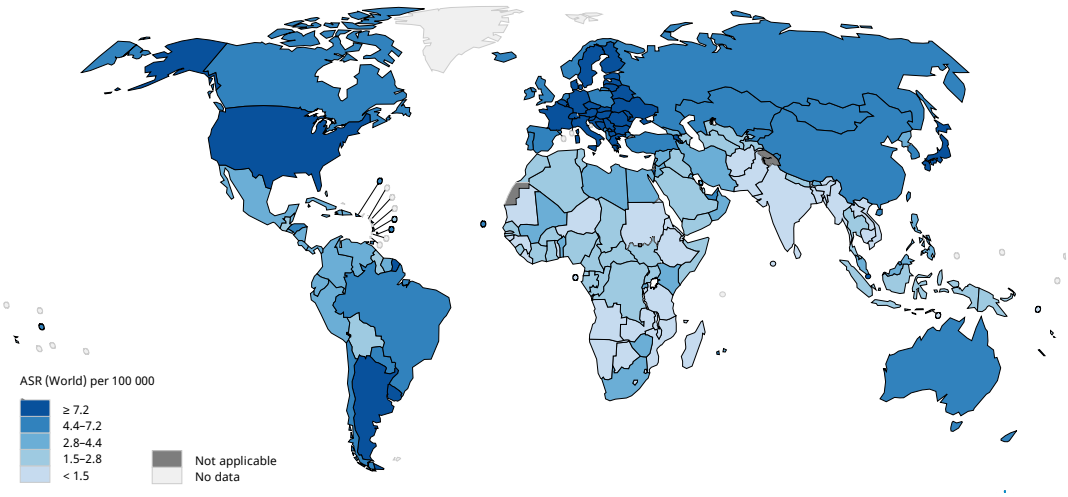


Figure 4. Estimated age-standardized incidence rates worldwide in 2018, for pancreas cancer

(adapted from Word Health Organization, IARC, 2018)

Incidence and mortality rates have been increasing in Europe (Bosetti *et al*., 2013), and North America (Kohler *et al*., 2015).

Seeing specifically where most of the new cases are estimated to have occurred in 2018 (figure 5), Asia stands out, making 46,7% of all new cases worldwide. Europe comes in second place (before North America) with 28.9% (Globocan, 2018).

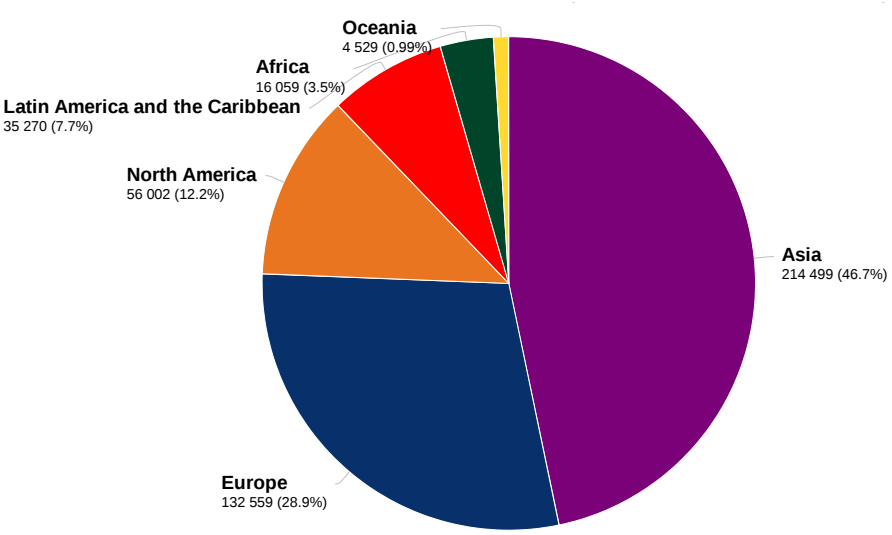


Figure 5. Estimated number of new cases of pancreatic cancer in 2018

(adapted from Globocan, 2018, http://gco.iarc.fr)

In 2018, despite not being one of the cancers with more new cases, pancreatic cancer counts to one of the eight with more deaths related to it, namely 4,6% of all cancer-related deaths (figure 6).

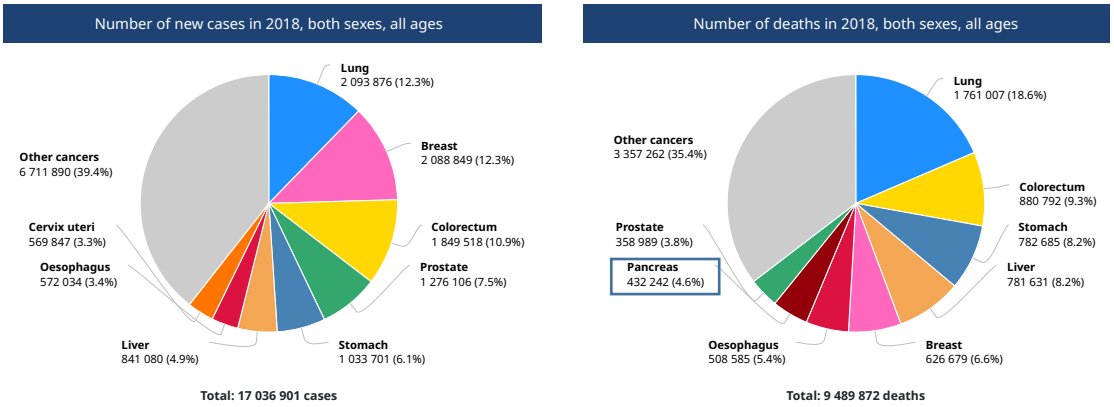


Figure 6. Number of new cases and deaths of cancer worldwide

(adapted from Globocan, 2018, http://gco.iarc.fr)

Comparing the number of incident cases and deaths in Europe and North America for Pancreatic Cancer (figure 7), the estimated age-standardized incidence is similar in both continents in 2018 (7.7 in Europe to 7.6 in North America) (Globocan, 2018).

Most pancreatic cancer patients are diagnosed at stage III or IV (79%), with only 21% having an early-diagnosis (stage I and II) (National Cancer Registration and Analysis Service, 2016, ISD Scotland, 2016).

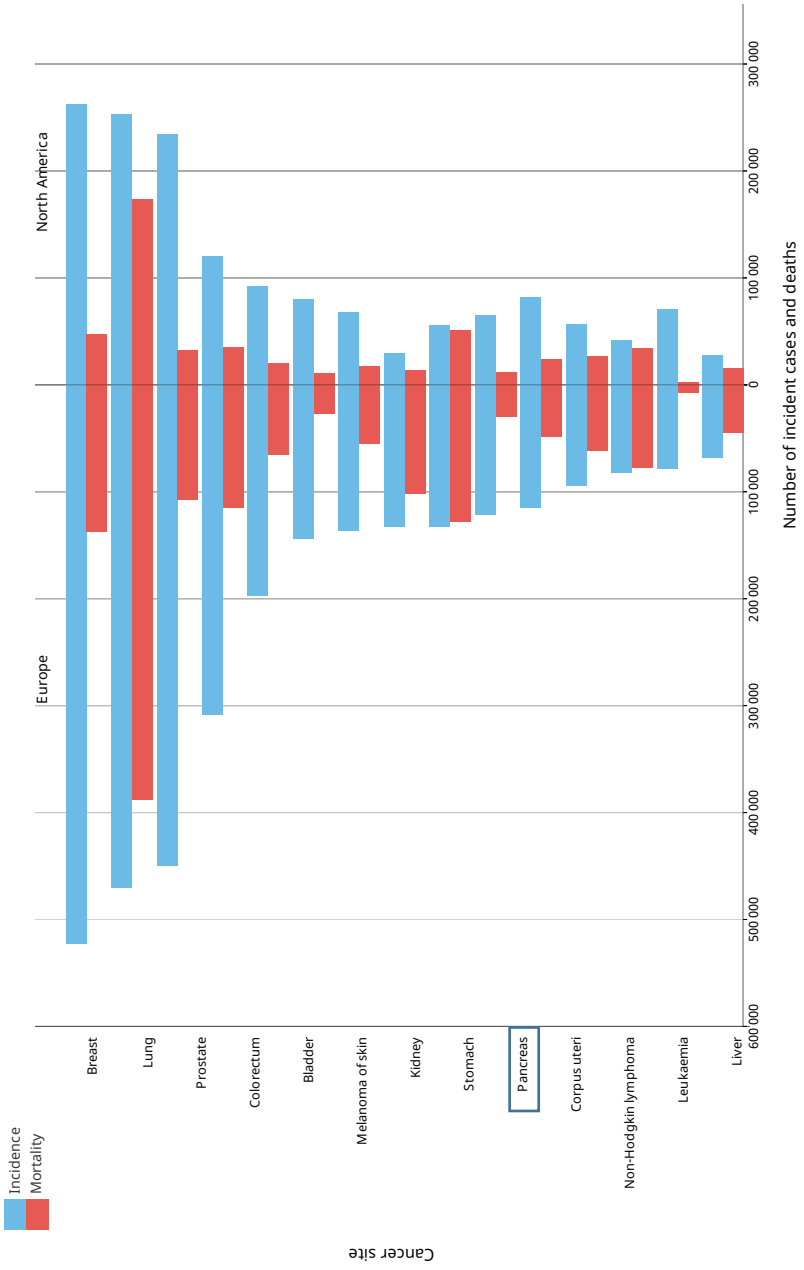


Figure 7. Estimated number of new cases and deaths in 2018, comparing Europe and America

(Globocan, 2018, http://gco.iarc.fr)

1.3 Etiology

Tumorigenesis is the progress of the cancer cells into a clinically significant disease, taking years. It is a complex process which can have innumerous causes and may involve lifestyle, namely diet and physical activity, environmental factors and host factors, like inheritance or epigenetic changes (figure 8). As for the host factors they can influence the probability of cancer development, with the accumulation of genetic damage over time. The interaction of these three types of factors over time is critical to the development (or not) of a malignancy (Annex 1).

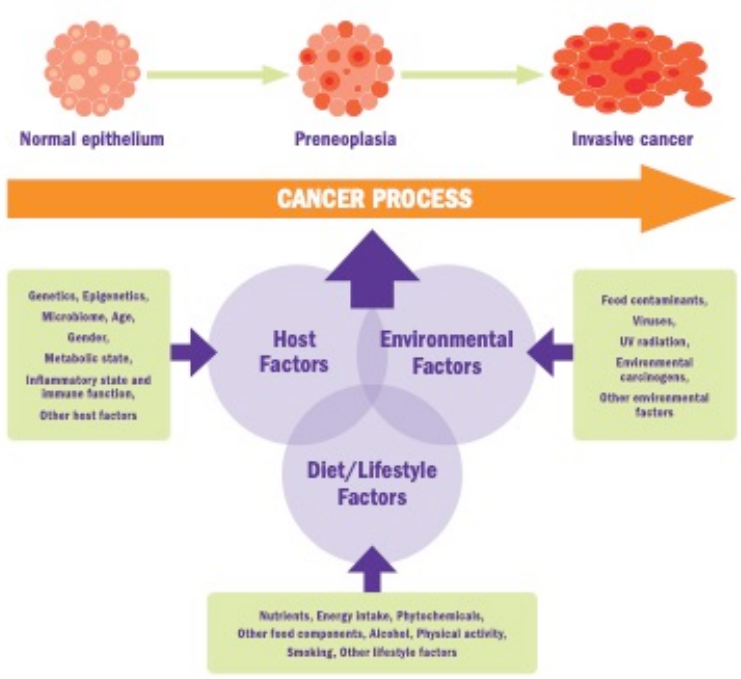


Figure 8. Factor affecting cancer process

(adapted from World Cancer Research Fund, 2018)

1.3.1 Non-modifiable risk factors

Pancreatic cancer, like all cancers and many other diseases, has a higher risk of occurring more frequently with **age** (World Cancer Research Fund, 2018). The risk increases especially after 65 years, with most diagnoses being made between the ages of 60 and 80 (Ferlay *et al*., 2012).

In addition to that, it also varies with **race**, as the black population has an increased risk by 1.5.

Regarding pancreatic cancer in specific, it has the highest incidence of **venous thromboembolism** in the first year of diagnosis, in comparison to the other neoplasms

(Nakchbandi *et al*., 2008). Patients with pancreatic cancer and venous thromboembolism also have a higher risk of poor prognosis and recurrence (Kondo *et al*., 2018) (table 2).

According to Klein *et al*. (2018), individuals with **non-0 blood group** also have an increased risk of pancreatic cancer.

Positive family history and some inherited genetic disorders also enhance the risk of pancreatic cancer, as are the Lynch syndrome, Peutz-Jeghers syndrome, familial atypical multiple mole melanoma syndrome, among others (Greer *et al*., 2007). Mutations in specific genes also increase de risk of developing this type of cancer (gene PRSS1, K-ras, p16, p53, BRCA2, ATM) (Slebos *et al*., 2000).

An established risk factor is also the history of **previous pancreatic diseases**, as the case of pancreatitis. Although a correlation of higher risk can be made, most patients with pancreatitis do not develop cancer (Zheng *et al*., 2013).

Considering that the **adult obtained height**, has as cause the number of cell divisions during fetal life and childhood, health and nutrition in childhood and age of sexual maturity, which changes hormonal microenvironment producing alterations in the level of growth factors (higher circulating levels of IGF-1 (Gunnell *et al*., 2001, Bray *et al*., 2006). Tall people have undergone more cell divisions, so there can be a higher potential for error during DNA replication, which can result in cancer (Le Roith *et al*., 2001) (table 2).

Table 2. Summary non-modifiable risk factors

Risk factor	Referance
Age (> 65 years)	Ferlay *et al*., 2012, World Cancer Research Fund/American Institute for Cancer Research, 2018
Black population	World Cancer Research Fund/American Institute for Cancer Research, 2018
Venous thromboembolism	Kondo *et al*. (2018)
Non-0 blood group	Klein *et al*. (2018)
Positive family history, some inherited genetic disorders	Slebos *et al*., 2000, Greer *et al*., 2007
Previous pancreatic diseases	Zheng *et al*., 2013
Bigger attained adult obtained height	Le Roith *et al*., 2001, World Cancer Research Fund/American Institute for Cancer Research, 2018

1.3.2 Modifiable risk factors

As already known, cigarette smoking (or chewing) is one of the biggest causes of cancer, especially lung cancer. **Tobacco** use is proven to be the leading cause of pancreatic cancer too (Secretan *et al*., 2009). It is estimated that 20% of pancreatic cancers are also caused by tobacco (IARC, 2012). The risk is increased by 74% for current smokers and 20% for former ones. Former smokers have an equal risk as non-smokers after 20 years (Lowenfels *et al*., 2006). According to Parkin *et al*. (2010), this risk may even be bigger. It is definitely the leading identified cause of pancreatic cancer. Tobacco and its smoke contain more than 7000 chemical compounds, many identified as carcinogens (World Cancer Report, 2014). These carcinogens contribute through many pathways to the development of the neoplasm: DNA binding, mutations, inflammation, oxidative stress and epigenetic changes.

Another cause of a major part of the pancreatic cancers is **body fatness** (especially abdominal) and **physical inactivity**. It also increases the risk of almost all gastrointestinal cancers. With an increase of body fatness, there can also occur **insulin resistance and diabetes** (Hursting *et al*., 2003), influencing levels of many other circulating hormones. This is why new-onset diabetes can be an early sign of pancreatic cancer (World Cancer Report, 2014). Due to the fact that insulin resistance is increasing in obesity, the pancreas compensates with and increased insulin

production. This **hyperinsulinemia** (Calle *et al*., 2004), is an environment propitious to carcinogenesis, discouraging apoptosis (World Cancer Research Fund, 2018).

Due to the fact that adipocytes (fat cells) produce pro-inflammatory factors (Calle *et al*., 2004), most obese individuals have a chronic inflammation (Rexrode *et al*., 2003), which in turn can also promote carcinogenesis (Loffreda *et al*., 1998).

By contributing to weigh control and influencing the overall metabolic state (World Cancer Research Fund, 2018), regular physical activity has proven to reduce the risks, as the reduction of sweetened food/ beverages and the consumption of vegetables, fruits and whole grains. Physical activity has also proven immunomodulatory effects (improves innate and acquired immunity) and promoting in this way a diminished risk of carcinogenesis (McTiernan *et al*., 2008, Fridenreich *et al*., 2010). Exercise also decreases oxidative stress improving in this way DNA repair mechanisms (Fridenreich *et al*., 2010).

Alcohol, if consumed in abuse (more than three drinks a day) (World Cancer Research Fund, 2018), has a dose-response association (Baan *et al*., 2009). Especially, but not only, ethanol is genotoxic contributing to carcinogenesis (World Cancer Report, 2014), being classified as a group 1 carcinogen (Duell *et al*., 2012) firstly in 1988, by the IARC. It is thought that its metabolites, like acetaldehyde might have a higher carcinogenic potential (World Cancer Research Fund, 2018).

The vast majority of the pancreatic juice samples studied by Maekawa *et al*. (2018) had **bacterial DNA**, namely *Enterococcus faecalis*, suggesting that it may be involved in the progression from pancreatitis to cancer. In 2018, about 16% of the total cancers were due to **infections with viruses, bacteria and microparasites**, as *Helicobacter pylori*, hepatitis B and C viruses (World Cancer Report, 2014). Through compromising the immune system, infection with HIV could also increase the risk of virus-related viruses.

Exposure to **all types of radiation**, like ionizing, ultraviolet and electromagnet one, can increase the risk of malignancies (World Cancer Report, 2014). This is both true for natural (like the sun) and man-made sources.

General **air pollution** from vehicles, households and a wide range of industries and drinking-water contamination may have carcinogens, as is the case of aromatic hydrocarbons (HAP), benzenes, asbestos, nitrates and nitrites and arsenic (World Cancer Report, 2014).

Plastic is produced using **Bisphenol A**. This is the reason why more than 90% of USA, Europe and Asia are exposed and probably contaminated by it through food or liquids (Vandenberg *et al*., 2010). In the 1980s it was established that Bisphenol A was not a robust carcinogen and recommended maximum daily safe dose was established to be

50 µg/kg/day (www.epa.gov/iris/subst/0356.htm). However, nowadays, with more than a hundred studies published since then, it is proven that this substance has an influence in the endocrine system (Keri *et al*., 2007), causing changes in the cancer cells at concentrations within the limits of exposure (Vandenberg *et al*., 2009). This evidence is however contested (Hengstler *et al*., 2011).

Pharmacological drugs, including the ones used as antineoplastic agents in cancer therapy could also potentially induce cancer development (as prevent it) (World Cancer Report, 2014). Due to its unspecific genotoxicity, antineoplastic drugs could be responsible for inducing second cancers in supposed cured patients (table 3).

Concluding, many cancers can be prevented, by not engaging in those modifiable risk factors. The recommendations by the World Cancer Research Fund can be seen figure 9.

Figure 9. Cancer Prevention Recommendations

(adapted from World Cancer Research Fund, 2018)

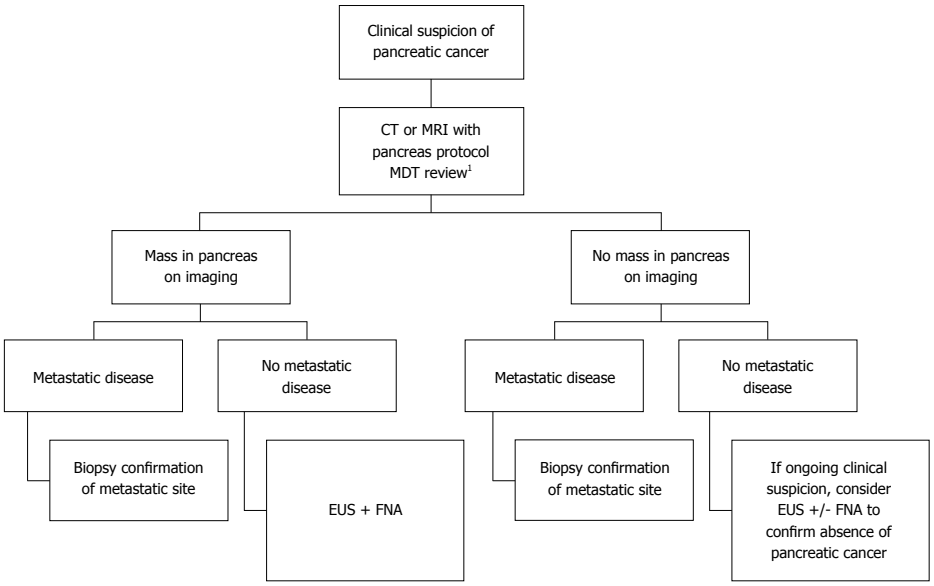
Table 3. Modifiable risk factors

Risk factor	Referance
Tobacco use	Secretan *et al*., 2009
Body fatness, physical inactivity	Hursting *et al*., 2003
Insulin resistance and diabetes	Hursting *et al*., 2003
Alcohol	World Cancer Research Fund/American Institute for Cancer Research, 2018
Infections with viruses, bacteria and microparasites	Maekawa *et al*. (2018), World Cancer Report, 2014
All types of radiation	World Cancer Report, 2014
Air pollution	World Cancer Report, 2014
Bisphenol A	Vandenberg *et al*., 2009
Pharmacological drugs	World Cancer Report, 2014

1.4 Current Diagnosis Methods

Currently, the only way to diagnose asymptomatic pancreatic cancer is using imaging techniques (Winter *et al*., 2006, Poruk *et al*., 2013). Imaging the pancreas is also used for cancer staging, monitoring the response to treatment and detection of metastatic lesions.

Among others, the techniques used are computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and endoscopic ultrasonography (EUS) (Morana *et al*., 2010, Appel *et al*., 2012, Fusaroli *et al*., 2012, Raman *et al*., 2012, Conrad *et al*., 2013) (figure 10).



CT: Computed tomography; MDCT: Multi-detector computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasound; FNA: Fine needle aspiration.

Figure 10. Algorithm for evaluation of a patient with suspicion of pancreatic cancer (adapted from Zhang *et al*., 2018a)

1.4.1 Multidetector computed tomography (MDCT)

For patients with suspicion of pancreatic adenocarcinoma MDCT is the most available and best-validated diagnostic method (Zhang *et al*., 2018a). In addition to that it also the cheapest method, safe and non-invasive (Ahn *et al*., 2009). It takes an image in which the disease and the rest of the pancreatic tissue can be well distinguished, as well as examination of distant disease (Brennan *et al*., 2007). For these reasons, it is normally the first method of diagnosis being used (National Comprehensive Cancer Network, 2017). Nevertheless, it is a nephrotoxic method which exposes the patient to radiation (Al-Hawary *et al*., 2014).

1.4.2 Magnetic Resonance Imaging (MRI)

MRI has a superior imaging, allowing theoretically a diagnosis in earlier stage, which has proven to be not true in the practical case (with the same sensitivity as CT scanning) (Treadwell *et al*., 2016). It is radiation free, but expensive and not possible if metal implants are present. For these reasons it normally is not the first choice (National Comprehensive Cancer Network, 2017).

1.4.3 Endoscopic Ultrasonography with fine needle aspiration (EUS)

EUS consists on an upper gastrointestinal endoscopic examination with an echoendoscope positioned in the stomach near the pancreas under sedation (Zhang *et al*., 2018a). It Is a safe, less invasive diagnosis method, having high sensitivity and detecting small lesions (Puli *et al*., 2013). It also allows the collection of a sample for biopsy (by needle aspiration) (Hewitt *et al*., 2012).

However, it is not always available, is operator dependent and it does not allow the detection of distant metastasis (Chen *et al*., 2012).

1.4.4 Positron Emission tomography (PET)

PET consists on enhancing the metabolism of glucose in cancer cells. These overexpress glucose transporter 1 and can accumulate glucose like this (Llop *et al*., 2018). For detection, Fluorine 18-fluorodeoxyglucose (FDG) is used. FDG is a glucose analogue, used as a radiotracer. FDG-PET has yet a low sensitivity and specificity, resulting often in false-negatives (due to hyperglycemia) and false-positives (as FDG levels increase in inflammations for example) (Matsumoto *et al*., 2013). PET has been showing to have better, more sensitive results for monitoring response to treatment in comparison to CT and for detecting recurrence (Sperti *et al*., 2010, Kinupe *et al*., 2017). Due to its wide anatomic coverage it is easier for metastatic detection in the entire body (Dibble *et al*. 2012, Lee *et al*., 2014).

1.4.5 Biopsy

Confirmation procedure, consisting on removing a small sample of tissue to be examined under microscope. This can occur by fine-needle aspiration (inserting a needle into the pancreas) or during EUS.

1.4.6 Blood test

The best, most invasive, easier and quicker way to diagnose is by a simple blood test, looking for biomarkers. Biomarkers can be used with different goals, namely early detection, therapeutic monitoring, follow-up after surgery and to guide treatment decisions (Llop *et al*., 2018).

For pancreatic cancer the only used is CA19-9, with no diagnostic goal though. It is currently FDA-approved for monitoring the disease.

1.5 Therapeutics

Currently the only curative option is surgery, which can only be performed in early-stage pancreatic cancer (Costello *et al*., 2012).

Unlike in most cancers, cytotoxic agents are still first-line treatment for pancreatic cancer (Shi *et al*., 2018). Nevertheless, there are not many pharmacological agents that can be used and therefore do not vary much with the type of pancreatic cancer.

According to Shi *et al*. (2018), the levels of CA19-9 should be monitored while choosing the therapeutic for patients with advanced pancreatic cancer.

1.5.1 Surgery

The only option that actually heals the patient. It can consist in removing only the part of the pancreas where the tumor is located or the entire pancreas. A person without pancreas can live normally needing insulin and enzyme replacement.

1.5.2 Chemotherapy

Consists in the use of toxic pharmacological substances injected or taken orally isolated or in combination with radiation therapy (Shi *et al*., 2018). In combination it is normally used in cases of wide spread pancreatic cancers or in cases where the tumor has first to be shrunken so that surgery is possible. It is also possible after surgery to prevent recurrence of the tumor.

1.5.3 Radiation Therapy

Cancer cells are destroyed with the use of highly energetic radiation, such as X-rays and protons. As chemotherapy, it can be used when surgery is not an option, to reduce the tumor before surgery or after surgery. Often in combination with chemotherapy.

2 Pancreatic Carcinogenesis

2.1 Pathology

Tumor mass consists of pancreatic stellate cells, immune cells, lymphatic and vascular endothelial cells, pathologically increased nerves and extracellular matrix (Duan *et al*., 2017).

The infiltrating ductal adenocarcinoma deduces an intense desmoplastic stromal reaction (Li *et al*., 2012, Rahib *et al*., 2014), creating a very complex tumor

microenvironment (Jiang *et al*., 2018). This complexity explains the lethality of this cancer, promoting its development (O'Neil *et al*., 2012, Duan *et al*., 2017). Due to its complexity, biopsies may mislead to false negative results (Olive *et al*., 2009).

Desmoplasia is the growth of fibrous or connective tissue. Although it may occur in benign circumstances like in scar tissue after surgery, it normally is associated with malignant neoplasms (causing dense fibrosis around it and invading healthy tissues like this) (Jiang *et al*., 2018).

Pancreatic stellate cells are thought to be the most responsible for the desmoplasia (Masamune *et al*., 2015). They occur normally in the pancreas, but they can be activated when in contact with pancreatic cells, synthesizing biologically active molecules (Pothula *et al*., 2016). They can also inhibit apoptosis and promote stem cells phenotypes of pancreatic cancer cells (Erkan *et al*., 2008), being therefore responsible for the resistance of pancreatic cancer to chemotherapy, distant metastasis and a poor prognosis (Hwang *et al*., 2008).

2.2 Genetics

Around 10% pancreatic cancer is of familial origin, meaning that some inherited mutated genes increase the risk of its development (Shi *et al*., 2009).

Exosome sequencing of ductal adenocarcinoma showed 16 significantly mutated genes (World Cancer Report, 2014). Including one oncogene (*KRAS*), three tumor suppressor genes (*CDKN2A*, *TP53*, *SMAD4*), *MLL3, ATM, TGFBR2, ARID1A*, and *SF3B1* (Jones *et al*., 2008).

Biankin *et al*. (2012) also discovered novel mutated genes: genes involved in chromatin modification (*EPC1* and *ARID2*) and DNA damage repair, and other mechanisms (*ZIM2*, *MAP2K4*, *NALCN*, *SLC16A4*, and *MAGEA6*).

Studying the mutated genes, does not only help in monitoring high-risk populations, but also in the development of personalized therapy, like the poly (ADP-ribose) polymerase inhibitors or mitomycin C for cancers with *BRCA2* or *PALB2* mutations (World Cancer Report, 2014).

3 Current State of the Art in Diagnostics

3.1 Novelties in Pancreatic Biomarkers

There is no current reliable early-state diagnostic biomarker for pancreatic cancer (Zhang *et al*., 2018b).

Due to the fact that most patients are only diagnosed with advanced pancreatic cancer, the samples to be studied for biomarkers come from them, making the identification of early-stage biomarkers more difficult (Resovi *et al*., 2018). This is why the use of in vivo models is fundamental.

Biomarkers are the most promising early-diagnosis method, due to its low cost, convenience, quickness and minimal invasiveness (Llop *et al*., 2018). However, it has been showed recently that no single biomarker could be reliable enough for cancer diagnosis, making a combination of biomarkers and incorporation of other clinical factors (as imaging techniques) a better approach (Capello *et al*., 2017, Chang *et al*., 2017). Figure 11 illustrates the potential early-stage pancreatic cancer biomarkers currently being studied.

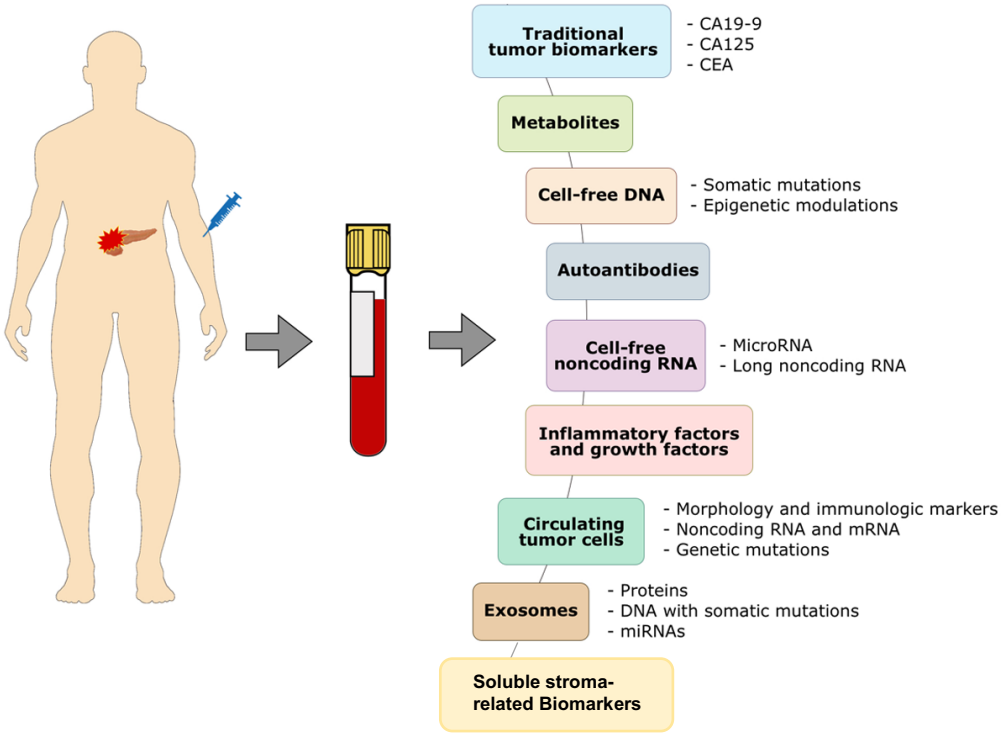


Figure 11. Overview of circulating biomarkers for early detection of pancreatic cancer (adapted from Zhang *et al*., 2018b)

3.1.1 Traditional tumor biomarkers

3.1.1.1 Carbohydrate Antigen 19-9 (CA19-9)

CA19-9 is a modified Lewis blood group carbohydrate antigen (Young *et al*., 2018), called siayil Lewis A (SLe^a). It is embedded on cell surface molecules, namely glycoproteins, gangliosides and mucins (Goh *et al*., 2017, Young *et al*., 2018), and is expressed in pancreatic and hepatobiliary diseases (Zhang *et al*., 2018a). It is the only FDA approved biomarker for pancreatic cancer (Locker *et al*., 2006), widely used with ELISA (Yue *et al*., 2011) but with many limitations (Wong *et al*., 2008).

It is used for monitoring the response of pancreatic cancer to treatment (Wong *et al*., 2008, Yue *et al*., 2011), as a prognostic biomarker (national cancer observatory) and for monitoring recurrence after surgical resection (Ballehaninna *et al*., 2012) having a lack of sensitivity and specificity for early-diagnosis (Huang *et al*., 2014). Their values to diagnosing pancreatic cancer vary according to different authors: 82 and 90% respectively (Llop *et al*., 2018) and according to Huang *et al*. (2014), it has a variable sensitivity of ~85% and specificity of ~85%. It has been shown that CA19-9 is upregulated up to two years before the diagnosis of pancreatic cancer, showing its good potential (O'Brien *et al*., 2015).

According to a study of Haab *et al*. (2015), most studies performed with CA19-9 do not use the same standards, varying from institution to institution. Feng *et al*. (2013), performed a large cohort, using the same rigorous conditions in every institution and key control groups. The goal was to understand if the different results obtained until that point, had divergent results due to the use of different standards. For this study they compared two antibody-based assay kits. As a global result, they reached similar AUC of 0.77 (area under the curve, it measures the accuracy with an AUC of 1 representing a perfect test) and comparable to the ones obtained in previous studies. However, these results varied a lot between patients, showing here a potential to be farther studied (Young *et al*., 2018).

As a prognostic biomarker it performs better in symptomatic patients (Bellehaninna *et al*., 2012, Huang *et al*., 2014). Nevertheless, it is not reliable when used alone (Singh *et al*., 2011).

According to Llop *et al*. (2018), it is not expressed in 10-20% of the Lewis antigen-negative population, due to a genetic deficiency of focusyltransferase activity (Capello

et al., 2017). In general, about 5-10% of the whole population does no express it (Herreros-Villanueva *et al*., 2016, Root *et al*., 2018).

In addition to that, it can lead to false-negatives, as only 65% of patients with pancreatic cancer have a high expression of CA19-9 (Gold *et al*., 2013).

It can also be falsely positive when other pathologies are present, like biliary infection, inflammation or obstruction, chronic pancreatitis and obstructive jaundice (Marrelli *et al*., 2009), hepatic and pancreatic cysts (Llop *et al*., 2018), other benign gastrointestinal conditions (Locker *et al*., 2006) or other cancers, like colorectal and breast (Llop *et al*., 2018). It has also racial and sex expression variations, being highest in Caucasians (Resovi *et al*., 2018).

In order to overcome these limitations and improve efficiency a combination of CA19-9 and other biomarkers has good potential (Faca *et al*., 2008, Chang *et al*., 2009, Brand *et al*., 2011 Gold *et al*., 2013, Lennon *et al*., 2014).

Many of these combinations with CA19-9 are being studied. The combination with **albumin and IGF** (Insulin-like growth factor 1) (Goh *et al*., 2017), can distinguish between pancreatic cancer and chronic pancreatitis with a high sensibility (93,6%) and high specificity (95%) (Park *et al*., 2012, Ferri *et al*., 2016).

Makawita *et al*. (2013), studied regenerating islet-derived 1 beta (REG1B), syncollin (SYCN), anterior gradient homolog 2 protein (AGR2), and lysyl oxidase-like 2 (LOXL2). The combination between **CA19-9, SYNC and REG1B** had the best results, with an AUC of 0.9.

In combination with **ICAM-1** (Intercellular Adhesion Molecule 1) and **OPG** (Osteoprotegerin), Brand *et al*. (2011), reported a sensitivity for diagnosis from 88% and a specificity of 94%. It is a highly selective combination that had negative results from almost all breast, lung and colorectal cancers.

In combination with **cathepsin D and MMP-7** (matrix metalloproteinase-7) it even has a higher AUC (Park *et al*., 2012).

Zeh *et al*. (2005) reported a combination of CA19-9 and serum cytokines (**HGF, MCP-1, IP-10, Eotaxin**) with very good results: sensitivity 85.7% and specificity of 92.3% to distinguish PC from healthy controls and sensitivity of 98%, specificity of 96.4% to distinguish PC from chronic pancreatitis. CA19-9 in combination with **albumin, C-reactive protein and interleukin 28** demonstrated sensitivity was 99.39% for all-stages, 96.10% for early-stage and 98.80% for advanced-stage pancreatic cancer at 90% specificity when discriminating between cancer and healthy individuals.

Cohen *et al*. (2017), studied the combination of four plasma proteins (**CA19-9, CEA (Carcinoembryonic antigen), HGF (Hepatocyte growth factor), OPN (osteopontin)) with ctDNA testing for KRAS mutations**. Although it outperformed

CA19-9 alone, it had a sensitivity of only 64%, making an optimization of the assays crucial to see if it could be an option to test furtherly.

Capello *et al*. (2017) researched already studied protein candidates to come with the best combination, namely **TIMP1 (TIMP metallopeptidase inhibitor 1), LRG1 (Leucine-rich alpha-2-glycoprotein 1) and CA19-9**, which outperformed CA19-9.

Combining CA19-9 with MUC5AC (Mucin 5AC), which in literature seems to have very good biomarker properties (is over-expressed in pre-cancerous lesions), Kaur *et al*. (2017), found it to have a better performance in ELISA.

The same was proven by Kim *et al*. (2017) for the combination **thrombo-spondin-2 (*THBS2*) and CA19-9.**

Sefrioui *et al*. (2017) studied a panel consisting in **CA19-9, ctDNA and CTC analysis**. In comparison with EUS-FNA alone it increased significantly the sensitivity and specificity.

According to Llop *et al*. (2018), combining **CA19-9 with miR-16 and miR196-a** it improves its performance to an AUC from 0,98.

All these combinations have still to be correctly validated first (table 4).

Table 4. Summary of CA19-9 combinations under study as early-stage diagnostic pancreatic cancer biomarkers

Biomarker Panel (concentration)	AUC, sensitivity, specificity	Referance
Carbohydrate Antigen 19-9 (CA 19-9)	AUC: 0.77; Sensitivity: ~85%; Specificity: ~85%	Feng *et al.* (2013) Huang et al, 2014
CA 19-9, albumin and IGF	Sensitivity: 93,6%; Specificity: 95%	Goh *et al*., 2017 Park *et al*., 2012 Ferri *et al*., 2016
CA 19-9, SYNC, REG1B	AUC: 0.9;	Makawita *et al*., 2013
CA 19-9, ICAM-1, OPG	Sensitivity: 78%; Specificity: 94%	Brand *et al*., 2011
CA 19-9, cathepsin D, MMP-7	AUC: 0.9 Sensitivity: 88%; Specificity: 80%	Park *et al*., 2012
CA 19-9, HGF, MCP-1, IP-10, Eotaxin	Sensitivity: 85.7%; Specificity: 92.3%	Zeh *et al*., 2005
CA 19-9, albumin, C-reactive protein and interleukin	Sensitivity: 99.39%; Specificity: 90%	Zeh *et al*., 2005
CA19-9, CEA, HGF, OPN with ctDNA testing for KRAS mutations	Sensitivity: 64%; Specificity: 99.5%	Cohen *et al*., 2017
CA19-9, TIMP1, LRG1	AUC: 0.95; Sensitivity:75%; Specificity: 95%	Capello *et al*., 2017
CA19-9, MUC5AC	AUC: 0.87; Sensitivity:83%; Specificity: 83%	Kaur *et al*., 2017
CA19-9, *THBS2*	Sensitivity:87%; Specificity: 98%	Kim *et al*., 2017
CA19-9, ctDNA and CTC analysis	Sensitivity: 78%; Specificity: 91%	Sefrioui *et al*., 2017
CA19-9, miR-16 and miR196-a	AUC: 0.98	Llop *et al*., 2018

3.1.1.2 Other conventional biomarkers

There are many other conventional biomarkers (Zhang *et al*., 2018b), namely CA242, CA72-4, CA125, CEA (Bunger *et al*., 2011). Although they have limited early-diagnostic potential (Zhang *et al*., 2015), they are promising for Lewis-negative population (in which CA19-9 has no utility).

An example of this high specificity seen for Lewis-negative patients are CEA and CA125 (98% and 93,8% respectively) (Luo *et al*., 2017).

However, this would imply first testing a possible patient regarding the state of the Lewis-group, which is not ideal

3.1.2 Metabolites

According to Hanahan *et al*. (2011), due to the fact that cancer cells can rewire metabolically, they can survive and proliferate even with oxygen and nutrient deficiency (called Warburg effect (Warburg *et al*., 1956)). In the specific case of pancreatic cancer, these cells survive in hypoxia, desmoplasia (Jiang *et al*., 2018) and hypovascularization.

A malignancy could therefore be diagnosticated in the presence of aberrant low-molecular weight substances (Denkert *et al*., 2012) deriving from this abnormal biochemical state (Vermeersch *et al*., 2013).

Because of the different demands of pancreatic cancer cells, especially metabolic ones, they can rewire the metabolism. This happens normally with a mutation of the oncogene KRAS (which is normally present in pancreatic cancer (Biankin *et al*., 2012)). These different metabolic demands are supported by the fibroblasts, which mediate metabolite exchange (Ozdemir *et al*., 2014, Rhim *et al*., 2014) and pancreatic stellate cells (Sousa *et al*., 2016).

Alterations in the metabolism include alterations in metabolic enzyme, accumulation of intermediates (Zhou *et al*., 2012), making amino acids, lipids, fatty acids and bile acids in serum potential biomarkers (Kobayashi *et al*., 2013, Ritchie *et al*., 2013, Zhang *et al*., 2013). One amino acid who is being studied is palmitic acid in serum. According to Di Gangi *et al*. (2016), it has been showed to have better results in early-diagnosis of pancreatic cancer than CA19-9 with an AUC of 1.0.

Mayers *et al*. (2014), also studied plasmatic amino acids, coming to the conclusion that an increase deriving from muscle catabolism could increase the risk of developing pancreatic cancer (Young *et al*., 2018).

Four other serum metabolites showed a higher sensitivity and lower false-negative rate when comparing with CEA and CA19-9, namely xylitol, 1,5-anhydro-D-glucitol, histidine, and inositol (Sakai *et al*., 2016).

There are extreme changes during different stages of pancreatic cancer. Metabolomics is therefore a sensitive indicator for monitoring pre-cancerous lesions and diagnosing early-stage (Yuan *et al*., 2016). For example, Kynurenate and methionine levels are only upregulated in pre-cancerous lesions and decreased in cancer.

Yuan *et al*. (2016) studied 82 metabolites in a prospective cohort study, concluding that two metabolites (isocitrate and aconitate) were associated with survival of pancreatic cancer patients. Both of these metabolites are intermediates of the tricarboxylic acid cycle. In addition to that, inherited ACO1 genotypes also influenced survival of patients.

Nowadays there have been identified already 50 differences in serum metabolites between pre-cancerous lesions and pancreatic cancer, making metabolites a good potential panel for biomarkers (LaConti *et al*., 2015).

Different enzymes and intermediates influence the concentration of a single metabolite, making the development of a panel with a combination of biomarkers crucial for a good diagnostic.

As for other potential biomarkers, they still have to be studied in larger populations and under standardized conditions (Zhang *et al*., 2018b) (table 5).

Table 5. Principal Metabolites being studied as early-stage diagnostic pancreatic cancer biomarkers

Biomarker Panel	AUC, sensitivity, specificity	Referance
Palmitic acid	AUC: 1.0; Sensitivity: 100%; Specificity: 100%	Di Gangi et al., 2016
Xylitol, 1,5-anhydro-D-glucitol, histidine, and inositol	Sensitivity: 84.1%; Specificity: 84.1%	Kobayashi *et al*., 2013

3.1.3 Cell-free DNA

By apoptosis or necrosis nucleic acids can be freed into the extracellular environment, originating cell-free DNA (cfDNA) (Zhang *et al*., 2018b). Cui *et al*. (2008), showed that patients with solid tumors normally have elevated levels of cfDNA.

Circulating tumor DNA (ctDNA) carries mutations corresponding to the somatic mutations in the primary tumor (Kinugasa *et al*., 2015), and it is believed that it correlates to its burden (Fleischhacker *et al*., 2007, Diaz *et al*., 2014, Newman *et al*., 2014), meaning that analyzing ctDNA from liquid biopsies could indicate tumor state and genetics. Bettegowda *et al*. (2014), showed that in stage I to III of pancreatic cancer, about 40% of patients have detectable ctDNA an about 90% for stage IV tumors.

Liquid biopsies are one of the most recent approaches in oncology, but still not suitable to replace tissue biopsies (Imamura *et al*., 2016, Pishvaian *et al*., 2016, Riva *et al*., 2016).

3.1.3.1 Somatic mutations

In pancreatic cancer, inactivating mutations in tumor suppressor genes, like CDKN2A, TP53, SMAD4, BRCA2 are found mostly in late states. In contrast to this, the most predominant genetic characteristics are KRAS mutations (Zhang *et al*., 2018b). They occur even in premalignant lesions and in high rate, being detected in serum, pancreatic juice and feces (Jones *et al*., 2008). However, it is not specific for pancreatic cancer as it also occurs in other diseases like chronic pancreatitis (Yanagisawa *et al*., 1993).

Its importance consists in controlling patients with pancreatic intraepithelial neoplasia. Almost 90% of these patients have a KRAS mutation with its rate correlating to the grade of the disease implying that a KRAS mutation is an early event during tumorigenesis (Kanda *et al*., 2012). There was also found a relation between KRAS mutations and clinical stage (Castells *et al*., 1999).

This potential biomarker still needs to be furtherly studied, as results of current studies do not always match (Mullcahy *et al*., 1998, Maire *et al*., 2002).

The detection rates vary from 35% of plasma samples of pancreatic cancer patients (Uemura *et al*., 2004) and 48% (Bettegowda *et al*., 2014) to 63% of pancreatic patients (Kinugasa *et al*., 2015).

Mutations in TP53 and SMAD4, in comparison with KRAS mutations, do not occur very often (Zhang *et al*., 2012). This is the reason why there are only a few studies on them. These mutations occur normally later, making them not suitable for early-stage diagnosis (Maitra *et al*., 2003) (table 6).

Table 6. Somatic Mutations of Cell-free DNA as early-stage diagnostic biomarkers

Biomarker Panel	AUC, sensitivity, specificity	Referance
KRAS mutation	Detection rate: 35-63% of pancreatic cancer patients	Uemura et al., 2004 Kinugasa et al., 2015
TP53 and SMAD4 mutations	Lower detection rates than KRAS mutation	Zhang et al., 2012

3.1.3.2 Epigenetic modulations

Epigenetic modifications of cfDNA (cell-free DNA), especially alterations in methylation pattern are very common in cancer, as it is the case of hypermethylation of tumor genomic DNA and hypermethylation of tumor suppressor genes (Zhang *et al*., 2018b). Sato *et al*. (2003), discovered several targets of abnormal DNA methylation in pancreatic cancer. UCHL1, NPTX2, SARP2, CLDN5, FOXE1, CDH3.

CD1D, KCNK12, CLEC11A, NDRG4, IKZF1, PKRCB and KRAS are currently under clinical study after resulting in 75% sensitivity and 95% specificity (Kisiel *et al*., 2015).

Other methylation biomarkers associated to the disease are ppENK, cyclin D2, sparc-7, Osteonectin and TSLC1. There has not been found further validation and clinical application for them yet (Fukushima *et al*., 2003, Matsubayashi *et al*., 2003, Sato *et al*., 2003).

There are, in addition to that, abnormal methylation profiles in specific cfDNA regions. Although P16 and proproenkephalin promoters are hypermethylated in plasmatic DNA, their detection rates are from about 30% and 25% accordingly (Jiao *et al*., 2007).

After analyzing 30 plasma samples, Liggett *et al*. (2010), combined some targets, particularly 14 gene promoters differentiating chronic pancreatitis patients from controls (sensitivity of 81,7% and specificity of 78%) and from pancreatic cancer patients (sensitivity of 91,2% and specificity of 90,8%) according to its methylation status. The panel included, CCND2 (cyclin D2), DAPK1 (death-associated protein kinase 1), ESR1 promA (estrogen receptor 1 promoter A), HMLH1 (human mutL homolog 1), MGMT (O-6-methylguanine-DNA methyltransferase), MUC2, (mucin 2, oligomeric mucus/gel-forming), MYOD1 (myogenic differentiation 1), CDKN2B (cyclin-dependent kinase inhibitor 2B), CDKN1C (cyclin-dependent
kinase inhibitor 1C), PGK1 (phosphoglycerate kinase 1), PGR-proximal (pro-gesterone receptor proximal promoter), RARb (retinoic acid receptor beta), RB1 (retinoblastoma 1), SYK (spleen tyrosine kinase).

A combination of CCND2, SOCS1 and THBS1 has also a very good potential for early-stage diagnosis, with a sensitivity of 76% and a specificity of 59% (Melnikov *et al*., 2009).

Studying the methylation status of BNC1 (Basonuclin 1) and ADAMTS1 (ADAM Metallopeptidase with Thrombospondin Type 1 Motif 1) in cfDNA could also be used for early-stage detection (81% sensitivity, 85% specificity) (Yi *et al*., 2013).

Still, epigenetic modifications need to be furtherly studied but have a very good potential. The incidence of aberrant DNA methylation at select cpg islands higher than incidence of genetic mutations and they have fewer false-negatives. This aberrant epigenetic alteration is an early event during tumorigenesis, it leads to gain/loss of function of critical molecules in cancer cells and the DNA methylation status is stable, making it easily detected with great sensitivity, even with contamination (Zhang *et al*., 2018b) (table 7).

Table 7. Principal methylation patterns of gene promoters in cfDNA as biomarkers

Biomarker Panel (Methylation status of gene promoters in cfDNA measured)	AUC, sensitivity, specificity	Referance
UCHL1, NPTX2, SARP2, CLDN5, FOXE1, CDH3. CD1D, KCNK12, CLEC11A, NDRG4, IKZF1, PKRCB and KRAS	Sensitivity: 75% Specificity: 95%	Kisiel et al., 2015
ppENK, cyclin D2, sparc-7, Osteonectin and TSLC1	With no validation yet	Fukushima et al., 2003, Ueki et al., 2002, Matsubayashi et al., 2003, Sato et al., 2003
CCND2, DAPK1, ESR1 promA, HMLH1, MGMT, MUC2, MYOD1, CDKN2B, CDKN1C, PGK1, PGR-proximal, RARb, RB1, SYK	Sensitivity: 91,2% Specificity: 90,8%	Liggett et al., 2010
CCND2, SOCS1, THBS1	Sensitivity: 76% Specificity: 59%	Melnikov et al., 2009
BNC1, ADAMTS1	Sensitivity: 81%; Specificity: 85%	Yi et al., 2013

3.1.4 Autoantibodies

Cancer patients normally develop an immune system dysfunction (Zhang *et al*., 2018b). Though the immune response is not strong enough to have clinical manifestations (Kobold *et al*., 2010), circulating autoantibodies could be potential biomarkers for diagnosing cancer.

In some types of cancer, including pancreatic, there are produced autoantibodies against tumor-associated antigens, ie. misfolded, overexpressed, aberrantly modified, ectopically expressed and mutated tumor proteins, (Desmetz *et al*., 2011, Kaur *et al*., 2012). These autoantibodies can indirectly reflect altered genetics and proteomics but can only be detected in low frequency (Zhang *et al*., 2018b). Due to tumor heterogeneity, detection is even more difficult.

Despite the fact that, in a study by Bracci *et al*. (2012), there were analyzed several autoantibodies, concluding that there are significant differences in expression levels in pancreatic cancer patients, their diagnostic value was poor with an AUC < 0.7.
Recently, the main focus on autoantibodies research has been Anti-mucin 1 antibodies (MUC1). MUC1 is a membrane associated glycoprotein that is overexpressed in some cancers, including pancreatic one and is associated with CA19-9 (Zhang *et al*., 2018b). In the last years, Gold *et al*. (2006), researched a monoclonal antibody against MUC1 with a sensitivity of 77% and a specificity of 95% for diagnosing pancreatic cancer.
Other autoantibodies being researched include autoantibodies to two acidic isoforms of glycolytic enzyme enolase (ENOA 1 e 2), which seem to be more frequent in patients with normal CA19-9 levels (Tomaino *et al*., 2011), Ezrin (Capello *et al*., 2013), vimentin isoform (Hong *et al*., 2006), and calreticulum isoforms (Hong *et al*., 2004) (table 8).
Neoantigen are new immunogenic protein sequences produced by malignancies. They are absent from the normal human genome and are primarily created by tumor-specific mutations in the genome. They could be potential targets for diagnoses and immunotherapy (Schumacher *et al*., 2015).

Table 8. Autoantibodies with potential as biomarkers

Biomarker Panel	AUC, sensitivity, specificity	Referance
Monoclonal antibody against MUC1	Sensitivity: 77% Specificity: 95%	Gold et al., 2006
ENOA 1 e 2	Sensitivity: 62% Specificity: 97%	Tomaino et al., 2011
Ezrin	AUC: 0.9 Sensitivity: 93.2% Specificity: 75.5%	Capello et al., 2013
Vimentin isoform	With no validation yet	Hong et al., 2006
Calreticulum isoforms	With no validation yet	Hong et al., 2004

3.1.5 Cell-free noncoding RNA (ncRNA)

Genes code proteins, but only about 2% of all genes. At least 75% of them do not encode anything, being called noncoding RNA (ncRNA) (Djebali *et al*., 2012). ncRNA include microRNA, small interfering RNA, piwi-interacting RNA, small Cajal body-specific RNA and long noncoding RNA (St Laurent *et al*., 2015).

It is suspected that ncRNA play regulatory roles, modifying gene expression at multiple levels through interactions with DNA, RNA and proteins during physiological processes and tumor development (Presner *et al*., 2011), especially miRNA and lncRNA that are specific of pancreatic cancer (Muller *et al*., 2015).
They can be detected in circulation, being potential biomarkers (Zhang *et al*., 2018b).

3.1.5.1 microRNA (miRNA)

miRNAs are small non-coding RNAs (18-25 nucleotides) that bind to complementary mRNA and inhibit gene expression. They regulate expression of more than 30% of human genes and may also function as tumor promoters or suppressors (Zhang *et al*., 2018b) as they can regulate gene expression. In oncogenesis there occurs a deregulation for which miRNAs may be responsible (Iorio *et al*., 2009, Li *et al*., 2013). Alterations in miRNA expression can, therefore, drive to malignancies (Lu *et al*., 2005).
On the blood, miRNA can be found circulating as free RNA attached to hAgo2 (an argonaute protein, that pairs to miRNA silencing it) (Fujita *et al*., 2004, Wakatsuki *et al*., 2005), and incorporated in exosomes (that protect it from degradation (Valadi *et al*., 2007)).
Due to their small size, stability, easy detection and convenient extraction they promise to be good biomarkers and to be furtherly studied. They are also deregulated in pancreatic diseases, making it able to discriminate pancreatic cancer from other malignancies (Bauer *et al*., 2012, Schultz *et al*., 2014).
Nonetheless, there is still no accepted internal control, studies lack a normalized standard and they can be bound to proteins or integrated in vesicles making their accurate quantification difficult. miRNA profiles are also very dynamic and easily and constantly influenced by other pathologies. Their origin and mechanisms are too still unclear (Zhang *et al*., 2018b). Because of these, developing better techniques for its evaluation could be crucial.
It was recently shown that miR-10, miR-21, miR-22, miR-155 when expressed in different rates could indicate pancreatic cancer (Roldo *et al*., 2006, Bloomston *et al*., 2007, Lee *et al*., 2007). They could have a higher diagnostic value than CA19-9.
According to a study performed by Schultz *et al*. (2014), where there were studied over 700 miRNAs, they found miR-145, miR-150, miR-223, miR-636 to be dysregulated in this malignancy. However, they proved to have an AUC (0.93), sensibility (0.85) and specificity (0.85) no superior than CA19-9 (AUC 0.9).
Another big, multicenter study focused on miR-486-5p (Xu *et al*., 2016), coming also to results comparable with the diagnostic value of CA19-9. Cao *et al*. (2016) also used

the potential of miR-486-5p. These authors tested two different panels containing this miRNA, namely miR-486-5p, miR-126-3p, miR-106b-3p (AUC: 0.89; sensitivity: 82.7%; specificity: 84.4%) and miR-486-5p, miR-126-3p, miR-106b-3p, miR- 938, miR-26b-3p, miR-1285 (AUC: 0.89; sensitivity: 82.3%; specificity: 81.4%).

Li *et al*. (2013), evaluated also over 700 circulating miRNAs. miR-1290 was the one with the best results, with and AUC of 0,96 for differentiating controls from pancreatic cancer patients (performing better than CA19-9) (Li *et al*., 2013).

Continuing to study and identifying altered levels of miRNA on the precursor lesions of pancreatic cancer could provide further comprehension of these processes (du Rieu *et al*., 2010, Xue *et al*., 2013). Also, miR-21 (du Rieu *et al*., 2010, Caponi *et al*., 2013), miR-155 (Ryu *et al*., 2010, Caponi *et al*., 2013), miR-196 (Slater *et al*., 2014), and miR-210 (Xue *et al*., 2013), are overexpressed in these precancerous lesions in tissue, serum, cyst fluid and stool making them promising biomarkers to be studied (Hernandez *et al*., 2016).

Schultz *et al*. (2014), identified two different miRNA panels that can differentiate healthy controls from pancreatic cancer patients. The first one consists of ten miRNAs (miR-26b, miR-34a, miR-122, miR-126, miR-145, miR-150, miR-223, miR-505, miR-636, miR-885.5p) and CA19-9. The second one includes four biomarkers, namely miR-145, miR-150, miR-223 and miR-636. For this second panel, the results were superior than those for CA19-9 alone (AUC: 0.93). Furthermore, it is also established that miR-216 and miR-217 are downregulated and miR-143, miR-145, miR-146, miR-148, miR-150, miR155, miR-196a, mir-196b, miR-210, miR-222, miR-223 and miR-31 are up-regulated in pancreatic cancer (Bloomston *et al*., 2007, Szafranska *et al*., 2007, Hanoun *et al*., 2010, Liffers *et al*., 2011).

miR-223, needing still further studying, might be indicated for early-stage diagnostic and predicting malignant potential from pre-cancerous lesions (Komatsu *et al*., 2015).

There are still very few studies associating miRNA levels and preneoplastic conditions (Komatsu *et al*., 2015). Studying patients with PanIN (pancreatic intraepithelial neoplasia) and IPMN (intraductal papillary mucinous neoplasm of the pancreas) and following them could help understand it.

That is why miRNAs are currently one of the most promising and most studied biomarkers for early-pancreatic cancer detection (Herreros-Villanueva *et al*., 2016). There is still a larger validation needed (table 9).

Table 9. Summary, miRNA as potential biomarkers

Biomarker Panel (concentration)	AUC, sensitivity, specificity	Referance
CA19-9, miR-16 and miR196-a	AUC: 0.98	Llop et al. (2018),
CA19-9, miR-145, miR-150, miR-223, miR-636	AUC: 0.93; Sensibility: 85%; Specificity: 85%	Schultz et al., 2014
miR-1290	AUC: 0.96	Li et al., 2013
CA19-9, miR-26b, miR-34a, miR-122, miR- 126, miR-145, miR-150, miR-223, miR-505, miR-636, miR-885.5p	AUC: 0.93; Sensitivity: 85%; Specificity: 85%	Schultz et al., 2014
miR-486-5p, miR-126-3p, miR-106b-3p	AUC: 0.89; Sensitivity: 82.7%; Specificity: 84.4%	Cao et al., 2016
miR-486-5p, miR-126-3p, miR-106b-3p, miR- 938, miR-26b-3p, miR-1285	AUC: 0.89; Sensitivity: 82.3%; Specificity: 81.4%	Cao et al., 2016

3.1.5.2 Long non-coding RNA (lncRNA)

LncRNAs consist in more than 200 nucleotides and can have or not a limited protein-coding capability (Ulitsky *et al*., 2013). They are limited to some specific cell types, playing crucial roles in tumorigenesis, because they modulate key pathways in transcriptional, posttranscriptional and epigenetic levels (Quinn *et al*., 2016).

Due to their tissue specificity, they could potentially be used to distinguish types of malignancies (Flippot *et al*., 2016).

Some lncRNAs are already used as diagnostic biomarkers in prostate, hepatocellular (Yuan *et al*., 2017), colorectal (Zhao *et al*., 2015) and lung cancer (Ren *et al*., 2013).

Regarding pancreatic cancer specifically, lncRNAs are diversely expressed according to tumor stage (primary or metastatic cancer) (Tahira *et al*., 2011).

For early-stage diagnosis, some lncRNAs that are dysregulated in pancreatic cancer comparing with healthy pancreatic tissues could be potentially used as biomarkers as are the cases of H19 (Scaiewicz *et al*., 2010), HOTAIR (Kim *et al*., 2013), HOTTIP (Fu

et al., 2017), MALAT-1 (Jiao *et al*., 2014), HULC (Peng *et al*., 2014), and GAS5 (Lu *et al*., 2013).
Fragments of the lncRNAs HOTTIP-005 and RP11-567G11.1, namely HDRF and RDRF should also be potential biomarkers, either each alone or in combination (Wang *et al*., 2015).

Most recently, Giulietti *et al*. (2018) identified eleven lncRNAs (A2M-AS1, DLEU2, LINC01133, LINC00675, MIR155HG, SLC25A25-AS1, LINC01857, LOC642852 (LINC00205), ITGB2-AS1, TSPOAP1-AS1 and PSMB8-AS1) that have different expression and promoter methylation levels and copy number alteration when comparing pancreatic cancer patients with healthy controls. Some of these, namely A2M-AS1, LINC01133, LINC00205 and TSPOAP1-AS1, could also be used as prognostic biomarkers.

Continuing to study lncRNAs may help understand the molecular pathogenesis of pancreatic cancer (table 10).

Table 10. lncRNA with potential as biomarkers

Biomarker Panel (concentration)	AUC, sensitivity, specificity	Referance
H19	Not validated yet	Scaiewicz et al., 2010
HOTAIR	Not validated yet	Kim et al., 2013
HOTTIP	Not validated yet	Fu et al., 2017
MALAT-1	Not validated yet	Jiao et al., 2014
HULC	Not validated yet	Peng et al., 2014
GAS5	Not validated yet	Lu et al., 2013
HDRF and RDRF	Not validated yet	Wang et al., 2015
A2M-AS1, DLEU2, LINC01133, LINC00675, MIR155HG, SLC25A25-AS1, LINC01857, LOC642852 (LINC00205), ITGB2-AS1, TSPOAP1-AS1 and PSMB8-AS1	Not validated yet	Giulietti et al., 2018

3.1.6 Inflammatory factors and growth factors

It is a known fact that inflammation plays an important role in tumorigenesis and metastasis that is why inflammatory factors like chemokines, cytokines and growth factors could be potential diagnostic biomarkers (Kaur *et al*., 2012).

Due to the fact that to the development of a tumor there needs to occur a growth factor stimulation and neovascularization (Zhang *et al*., 2018b), pancreatic cancer patients have high serum levels of two growth factors, namely VEGF and bFGF (Pistol-Tanase *et al*., 2008). These high levels also correlate to tumor size.

Shaw *et al*. (2014), studied IP-10, IL-6, PDGF and CA19-9 improving its diagnostic performance.

After comparing several inflammatory factor profiles between healthy and pancreatic cancer patients, Wingren *et al*. (2012) found that several ones were overexpressed: C1 esterase inhibitor, C3, C5, CD40, CD40 ligand, factor B, GLP-1, IFN-γ, igm IL-10, IL-11, IL-12, IL-13, IL-16, IL-18, IL-1-ra, IL-1α, IL-3, IL-5, IL-6, IL-7 and IL-8, integrin-

α-11, procathepsin W, sialyl Lewis x, TGF-β1, TNF-α and VEGF, based on which he constructed a panel of 25 biomarkers for early-stage diagnosis (AUC 0.95).

Yoshinaga *et al*. (2018), researched angiopoietin-like protein 2 (ANGPTL2), which is related to chronic inflammation and Type 2 diabetes mellitus, regarding its capability to early-diagnosis. The serum concentration of ANGPTL2 was significantly higher in pancreatic cancer patients than in healthy controls making it a potential biomarker to be furtherly studied.

Macrophage Inhibitory Cytokine 1 (MUC-1) seems promising, showing high levels in pancreatic adenocarcinoma (O'Brien *et al*., 2015). It also proved to have a good diagnostic accuracy when combined with CA19-9 (Koopmann *et al*., 2004, Ni *et al*., 2005) (table 11).

Table 11. Summary of inflammatory and growth factors

Biomarker Panel (concentration)	AUC, sensitivity, specificity	Referance
IP-10, IL-6, PDGF and CA19-9	Not validated yet	Shaw et al., 2014
C1 esterase inhibitor, C3, C5, CD40, CD40 ligand, factor B, GLP-1, IFN-γ, igm IL-10, IL-11, IL-12, IL-13, IL-16, IL-18, IL-1-ra, IL-1α, IL-3, IL-5, IL-6, IL-7 and IL-8, integrin-α-11, procathepsin W, sialyl Lewis x, TGF-β1, TNF-α and VEGF	AUC: 0.95	Wingren et al., 2012
ANGPTL2	Not validated yet	Yoshinaga et al., 2018
MUC-1	Not validated yet	O'Brien et al., 2015
MUC-1, CA19-9	Not validated yet	Koopmann et al., 2004, Ni et al., 2005

3.1.7 Circulating tumor cells (CTCs)

Circulating tumor cells are tumor cells that can enter circulation (Herreros-Vilanueva *et al*., 2016). They can be detected in early stages (before imaging techniques (Rhim *et al*., 2014)), in bloodstream as they leave primary lesion in the beginning of tumor development (Pantel *et al*., 2004, Husemann *et al*., 2008, Rhim *et al*., 2012). Their existence is already known for a century (Riva *et al*., 2016). Due to the fact that they carry tumor markers on the surface and somatic mutations, they can be used for liquid and quick biopsies (O'Flaherty *et al*., 2012).

CTCs can be identified in at least 40% (with some studies suggesting that it can reach even 100% of patients with pancreatic cancer) (Iwanikci-Caron *et al*., 2013).
They began to be studied only recently for the reason that the technological advances needed to its analysis where only made in the last times. CellSearch platform enabled the selecting of CTCs from blood cells, as they express cell adhesion molecule (EpCAM) (Nagrath *et al*., 2007).
It is believed that they are responsible for metastasis development (Herreros-Vilanueva *et al*., 2016). Nevertheless, its isolation and enrichment are extremely difficult making its detection extremely rare in pancreatic cancer (Rhim *et al*., 2012).
The results of the studies of CTCs still remain debatable. According to a study by Albuquerque *et al*. (Albuquerque *et al*., 2012), 47% of pancreatic cancer patients presented CTCs. In contrast to this, another study (Bidard *et al*., 2013), demonstrated that there were detected by CellSearch platform CTCs in only 5% of patients.
Yang *et al*., (Gao *et al*., 2016), analyzed CTCs with another technique, consisting in a platform with subtraction, enrichment and imunnostaining-fluorescence *in situ* hybridization. It showed an 88% sensitivity, 90% specificity and an early-detection rate of 12/13.
It can therefore be concluded that CTCs are promising biomarkers, even if only as prognostic biomarkers (Riva *et al*., 2016). There must still be developed a standardized detection method and a large-scale validation (Zhang *et al*., 2018b).

3.1.8 Exosomes

Exosomes are small vesicles containing nucleic acids and proteins (Skog *et al*., 2008, Trjkovic *et al*., 2008). They are secreted by almost all cells, including cancer cells, playing an important role in intercellular communication, tumorigenesis and metastasis (Melo *et al*., 2014, Robbins *et al*., 2014 Hoshino *et al*., 2015).

They are composed externally by a lipid bilayer and do not have any cellular organelles (Azmi *et al*., 2013). When released, they are very stable in the extracellular environment and can also be taken up by other cells, exchange material or information between cells (Lo Cicero *et al*., 2015), and promote tumorigenesis (Charrier *et al*., 2014). The main circulating DNA is associated with exosomes (Kahlert *et al*., 2014).
Exosomes contain many proteins, nucleic acids and lipids from cancer cells making them good candidates for diagnostic biomarkers. They are stable in almost all body fluids. What makes them good candidates as biomarkers is that they enter circulation in early stages of the tumorigenesis. In the case of pancreatic cancer, metastasis can even occur at early stage (Zhang *et al*., 2018b).
However, the isolation of malignant exosomes is challenging, making the development/ improvement of techniques crucial (table 12).

3.1.8.1 Proteins

Glypican-1 (GPC1) is a membrane-anchored protein that is overexpressed in pancreatic cancer and precancerous lesions. For differentiating healthy controls, it had a perfect AUC (1.0) (Melo *et al*., 2015). Comparing sensitivities GPC1 from serum exosomes has a better sensitivity than from whole serum. Meanwhile there have already been enough studies proving its significance for diagnosing pancreatic cancer (Zoller *et al*., 2013).
Combining five proteins (CD44v6, Tspan8, epcam, MET, and CD104) and four miRNAs (miR-1246, miR- 4644, miR-3976, and miR-4306) in circulating exosomes it could discriminate pancreatic cancer with a sensitivity of 1.0 and a specificity of 0.8 (Madhaven *et al*., 2015).

3.1.8.2 DNA with somatic mutations

Circulating exosomes carry a large amount of tumor DNA with its variations, somatic mutations and expressed fusion genes (San Lucas *et al*., 2016).
As already approached, during malignancies, there occur and are identifiable KRAS and TP53 mutations. These are also present in circulating exosomal DNA, with a detection rate of almost 40% for pancreatic cancer patients, almost 30% in precancerous lesions and only 3% in healthy controls (Yang *et al*., 2017).
The detection of KRAS mutations is higher in exosomes than in cfDNA (Allenson *et al*., 2017).

3.1.8.3 miRNAs

Ding *et al*. (2015) disclosed that exosomes from pancreatic cancer transfer miRNAs to dendiritic cells by miR-212 to induce immunotelarance.

Regarding the detection miRNA in circulating exosomes, pancreatic cancer patients have different levels of miR-10b, miR-20a, miR-21, miR-30c, miR-106b and miR-let7a. These levels normalized after tumor resection (Lai *et al*., 2017).

Table 12. Principal exosomes

Biomarker Panel (concentration)	AUC, sensitivity, specificity	Referance
Proteins		
GPC1	AUC: 1.0	Melo *et al*., 2015
CD44v6, Tspan8, epcam, MET, CD104, miR-1246, miR- 4644, miR-3976, miR-4306	Sensitivity: 100% Specificity: 80%	Madhaven *et al*., 2015
DNA with somatic mutations		
TP53 **KRAS**	Detection rate: 40% of pancreatic cancer patients	Yang *et al*., 2017
miRNAs		
miR-10b, miR-20a, miR-21, miR-30c, miR-106b and miR-let7a	Aberrant levels	Lai *et al*., 2017

3.1.9 Soluble stroma-related Biomarkers

Stromal modifications occur early in tumorigenesis and persist. Tumor initiation, growth and metastasis relies on the creation of a favorable microenvironment, including cancer cells, stroma and immune/ inflammatory cells (Erkan *et al*., 2013), reason why, stroma-related circulating molecules make good potential early-stage biomarkers.

Resovi *et al*. (2018), studied 38 potential molecules (extracellular matrix proteins and proteolytic fragments, matrix-degrading enzymes and their inhibitors, growth factors, antiangiogenic factors, adhesion molecules (Yu *et al*., 2005; Bloomston *et al*., 2006;

Faca *et al*., 2008; Kojima *et al*., 2008; Fiedler *et al*., 2009; Rong *et al*., 2010; Xue *et al*., 2010; Pan *et al*., 2011)).

Of these, seven molecules were significantly up-regulated in pancreatic cancer when compared with healthy controls, namely MMP7 (AUC 0.98) with an excellent discriminatory ability, CCN2 (AUC 0.86) similar to CA19-9 (AUC 0.87), TIMP1 (AUC 0.82), IGFBP2 (Insulin Like Growth Factor Binding Protein 2) (AUC 0.82), TSP2 (Thrombospondin-2) (AUC 0.78), sICAM1 (Soluble intercellular adhesion molecule-1) (AUC 0.77) and PLG (plasminogen) (AUC 0.66).

Resovi *et al*. (2018), also studied combination of these seven potential biomarkers, concluding that the panel consisting of MMP7, CA19-9 and CCN2 had the best results (AUC 0.94).

MMP7 (matrilysin) is an inducer of acinar-to-ductal metaplasia (Crawford *et al*., 2002; Sawey *et al*., 2007), that is upregulated in precancerous (pancreatic) lesions. There is known to be high MMP7 plasma levels in association with early tumor progression in precancerous and earl-stages (I, II), reason why it has value as a potential early diagnostic biomarker.

CCN2 (connective tissue growth factor (CTGF)) is overexpressed by activated pancreatic stellate cells in the earliest stages of pancreatic cancer. It promotes local desmoplasia, tumor survival, and metastasis (Leask *et al*., 2009) (table 13).

Table 13. Soluble stroma-related biomarkers (summary)

Biomarker Panel (concentration)	AUC, sensitivity, specificity	Referance
MMP7	AUC 0.98	Resovi et al., 2018
CCN2	AUC 0.86	Resovi et al., 2018
TIMP1	AUC 0.82	Resovi et al., 2018
IGFBP2	AUC 0.82	Resovi et al., 2018
TSP2	AUC 0.78	Resovi et al., 2018
sICAM1	AUC 0.77	Resovi et al., 2018
PLG	AUC 0.66	Resovi et al., 2018
MMP7, CA19-9 and CCN2	AUC 0.94	Resovi et al., 2018

3.2 Glycosylation in Cancer

Glycosylation is the enzymatic process in which saccharides are linked through a glycosydic linkage to other saccharides, proteins or lipids (Fuster *et al*., 2005, Moremen *et al*., 2012). Glycoproteins consist on one or more glycans covalently attached to a polypeptide backbone. This linkage usually occurs via nitrogen or oxygen linkages, reason why they are named *N*-glycans or *O*-glycans, respectively (Varki *et al*., 2009, Bennett *et al*., 2012).

Glycan structures of glycoconjugates such as glycoproteins are modified in diseases, like inflammation, infectious diseases, diabetes, neurodegeneration and cancer (Kuzmanov *et al*., 2013, Hart *et al*., 2015, Munkley *et al*., 2016, Kailemia *et al*., 2017). Alterations in glycosylation in cancer have already been described for over 60 years (Ladenson *et al*., 1949, Hakomori *et al*., 1968). One of the main features in tumorigenesis seem to be aberrant glycosylation, as glycans are involved in main regulatory mechanisms, like protein folding and clearance rates, cell signaling (Boscher *et al*., 2011, de-Freitas-Junior *et al*., 2013, Gomes *et al*., 2013, Takeuchi *et al*., 2014), angiogenesis, differentiation, cell growth, cell-matrix interactions (Zhao *et al*., 2008), immune modulation (Zhao *et al*., 2008), tumor cell dissociation, invasion,

epithelial-mesenchymal transition (EMT), and metastasis (Pinho *et al*., 2015, Llop *et al*., 2018). Glycans alter protein conformation and structure, modulating in this way the functional activity of the protein (Helenius *et al*., 2001).

For a tumor to develop it has to gain the ability to overcome cell–cell adhesion and to be able to invade surrounding tissue. Epithelial cadherin (E-cadherin) is a transmembrane glycoprotein (Pinho *et al*., 2011) and a major epithelial cell–cell adhesion molecule in cancer (Paredes *et al*., 2012). Glycans can have a crucial effect on tumor cell–cell adhesion by directly interfering with E-cadherin functions. In cancer there is also a mutual regulatory mechanism between E-cadherin-mediated cell–cell adhesion and its glycosylation leading either to tumor suppression or tumor metastasis (Gu *et al*., 2009, Pinho *et al*., 2013).

Cancer cells have a different metabolism than normal ones. There is a shift from oxidative phosphorylation to aerobic glycolysis, called the Warburg effect (Warburg *et al*., 1956). This high glucose uptake is used to cope with the increased energetic and biosynthetic needs to generate a tumor. For this purpose, cancer cell can also upregulate glutamine uptake. As a result, the cytoplasm of cancer cells is abundant in glucose. It contributes to increased glycolysis and increases the flux into the metabolic branch pathways.

3.2.1 Glycomic aberration vs. biomarkers

As alterations in glycosylation regulate the development and consequent progression of cancer, they are potential biomarkers for early-diagnosis and targets for therapeutic strategies (Hakomori *et al*., 2002, Fuster *et al*., 2005, Taniguchi *et al*., 2009, Reis *et al*., 2010, Freeze *et al*., 2013, Pinho *et al*., 2013). Alterations in glycosylation have a major heterogeneity. This is because aberrant glycan modifications are protein-specific, site-specific (meaning that different sites on the protein can be differentially glycosylated) and cell-specific (Pinho *et al*., 2015). There are two main mechanisms for tumor-glycosylation firstly postulated by Hakomori and *et al*. (1983), namely incomplete synthesis and neo-synthesis process. Regarding incomplete synthesis process, it occurs normally in early-stages of the malignancy. It is a consequence of the damage of the normal synthesis of complex glycans and leads to the biosynthesis of truncated structures (abnormally shortened glycans), like sialyl Tn. In contrast to this, neo-synthesis process more often arises in advanced stages. It regards the induction of genes involved in expression of carbohydrate determinants (like *de novo* expression of antigens like sialyl Lewis). These modifications can be detected in biological fluids of the cancer patients, as a result of the aberrant glycoproteins being

shed into the bloodstream (Peracaula *et al*., 2008, Meany *et al*., 2011). All of the nine proteins (Polasnki *et al*., 2007) approved as cancer biomarkers, are glycosylated proteins (Schiess *et al*., 2009, Diamandis *et al*., 2010, Füzéry *et al*., 2013, Kuzmanov *et al*., 2013, Pavlou *et al*., 2013) showing the importance of them.

There is a major glycan diversity, due to many factors (Pinho *et al*., 2015). First of all, monosaccharides can have innumerous compositions, as is the case of galactose or *N*-acetylgalactosamine for example. Monosaccharides can also be linked in different ways, with the Carbon atoms involved in the linkage varying (C1-C3 or C1-C4). In addition to that, monossacharides have a terminal aldehyde/ketone. This hydroxy group can either be in α or β configuration, changing accordingly the anomeric state of the monosaccharide. Branching structures, or in its linkage to their aglycone (non-glycosyl) part (Cummings *et al*., 2009, Varki *et al*., 2009). Concerning abnormal glycosylation, this too has innumerous causes. In the first place it can be due to under- or overexpression of glycosyltransferases (enzymes that catalyze the transfer of the glycosyl-group to the aglycone (lipids, proteins, carbohydrates)). This occurs because of a dysregulation at transcriptional level (Kannagi *et al*., 2009 Hatano *et al*., 2011, Pinho *et al*., 2012), a dysregulation of chaperone function (Schietinger *et al*., 2006, Aryal *et al*., 2010) (chaperones stabilize unfolded proteins) or altered glycosidase activity (Kakugawa *et al*., 2002) (enzymes that catalyze hydrolysis of glycosylic linkages, separate glycosyl-group from aglycone). Furthermore, abnormal glycolysation can be caused by changes in tertiary conformation of the peptide backbone and of the nascent glycan chain. Another possible cause consists on the variability of acceptor substrates in addition to the availability and abundance of sugar nucleotide donors and cofactors (Kumamoto *et al*., 2001). This means that there is an alteration in glycemic expression, because there is a whole panoply of ligands that can be linked during glycosylation. Besides, glycosyltransferases can be mislocated (in the Golgi apparatus) or there can be a change in its activity leading to synthesis of immature core glycan structures (Kellokumpu *et al*., 2001, Marcos *et al*., 2004, Brockhausen *et al*., 2006, Gill *et al*., 2010).

There are many studies regarding serum glycoproteins with altered glycan chains in pancreatic cancer patients, especially altered N-glycan patterns in tumors (Arnold *et al*., 2008). They do not have sufficient specificity though, as many of these N-glycosylation also occur in benign conditions. Llop *et al*. (2018) propose combining aberrant glycosylation with the altered protein levels in order to improve its performance in diagnosing pancreatic cancer (Kailemia *et al*., 2018).

Almost all tumor cells tend to be affected by aberrant glycosylation, making these changes in glycosylation more pronounced than alterations in protein expression.

With progression of the cancer, glycans expressed are continuously and rapidly changing (Pinho *et al*., 2015, Munkley *et al*., 2016) and alterations become more marked the more aggressive the tumor becomes. This is why glycan alterations should be more reliable biomarkers, also in predictive value (Silva *et al*., 2015).

Nonetheless, the analysis of the glycoproteome in pancreatic cancer has not been extensively studied yet. One possible reason for this is that the analysis of the glycoproteome is much more complex than the one of the proteome, as there is no template (like the amino acid sequence coded in the genome for proteins) for glycans (Llop *et al*., 2018) and there is a major amount of heterogeneity.

There are four main cancer-associated glycosylation processes (Stowell *et al*., 2015): sialylation (siayil-Lewis= CA19-9 antigen), fucosylation, increased GlcNAc-branching of N-glycans and over-expression of truncated mucin-type O-glycans.

Sialylation plays a critical role in cellular recognition, cell adhesion and signaling. There are two major sialylated antigens associated with cancer, namely SLe^a, SLe^x. Both of them are correlated with a poor prognosis (Amado *et al*., 1998, Baldus *et al*., 1998).

SLe^x is a ligand for selectins (Rosen *et al*., 1994). Selectins are vascular cell adhesion and belong to a family of C-type lectins. During inflammation selectins mediate the initial attachment of leukocytes to the endothelium during the process of leukocyte extravasation (Rosen *et al*., 1994). In cancer, SLe^x interactions with selectins regulate the metastatic cascade, determining the malignant behavior and development of metastasis (Nakamori *et al*., 1993).

Ceruplasmin is an acute-phase protein, that has an increased siayil-Lewis (SLe^x) level in pancreatic cancer. In a big cohort study of pancreatic cancer serum samples, the ratio of SLe^x on ceruplasmin related to the ceruplasmin level showed to be increased in pancreatic cancer in comparison to chronic pancreatitis (Balmaña *et al*., 2015). SLe^a is detected by serological assay CA19-9.

Concerning fucosylation, it is catalyzed by numerous fucosyltransferases, including FUT3, which is a Lewis Gene (Le). Fucosylation is divided in terminal and core fucosylation. Terminal fucosylation originates specific Lewis blood-group antigens (Le^x and Le^y and Le^a and Le^b). Contrarily, core fucosylation consists in the addition of α1,6-fucose to the innermost GlcNAc residue of *N*-glycans through the action of Fuc-TVIII (encoded by *FUT8*) (Carvalho *et al*., 2010).

Otherwise, branching and bisecting GlcNAc N-glycans is also commonly present in malignancies. In these cases, there is an increase in the expression of complex β1,6-branched *N*-linked glycans 2 (Dennis *et al*., 1987). Branched *N*-glycans are further modified by β1,4-GalTs, elongated with poly-*N*-acetyllactosamine and further capped

with sialic acid and fucose (Dennis *et al*., 1987). This poly-*N*-acetyllactosamine structure is a ligand for galectins (Di Lella *et al*., 2011). Galectins have important roles in cancer, contributing to neoplastic transformation, tumor cell survival, angiogenesis and tumor metastasis (Croci *et al*., 2014).

α-fetoprotein (AFP) is a glycoprotein fucosylated and in overexpression in hepatocellular carcinoma and other benign hepatic diseases. Detecting simultaneously both its overexpression and its fucosylation (ratio fucosylated AFP/ total AFP) is approved as a biomarker for risk assessment of risk patients for the development of hepatocellular carcinoma (Li *et al*., 2001, Wang *et al*., 2009). With the same thought, there exists also biomarker with changes in fucosylation for prostate cancer (Tabarés *et al*., 2006, Sarrats *et al*., 2010) used for screening high-risk populations (Llop *et al*., 2016, Ferrer-Batallé *et al*., 2017). Both biomarkers already used in two types of cancer show the great potential of fucosylation in early-stage diagnostics of cancer.

Regarding the glycosylation profile of pancreatic carcinomas, there is an increase of branching of N-linked oligosaccharides (Zhao *et al*., 2007), in protein fucosylation and sialylation in pancreatic cancer serum. Another main feature consists on the increment in Lewis and blood group glycans (Pour *et al*., 1988, Pérez-Garay *et al*., 2013). According to Remmers *et al*. (2013), there also is also present a truncated O-linked glycosylation resulting in the Tn and sialyl-Tn antigens.

As a starting point for the search of glycoproteins as biomarkers normally count whole serum samples or serum after removing the most abundant ones are removed, as they should hinder the detection aberrant glycosylation patterns that should exist in low concentration (Llorens *et al*., 2018). There can be used lectins or antibodies against specific glycan structures or mass-spectrometry (MS) to identify the different glycosylation of the glycoproteins.

Normally the strategy consists on analyzing alterations in branching, fucosylation (Tan *et al*., 2015, Terao *et al*., 2015) sialylation (Kontro *et al*., 2014) and specific N-glycosylation occupancy (Pan *et al*., 2014).

There is an increase in fucosylation and sialylation described for pancreatic cancer (Zhao *et al*., 2007, Li *et al*., 2009).

Two acute-phase proteins (haptoglobin (HPT) and α-1-acid-glycoprotein (AGP)) are increased in core fucosylation in pancreatic cancer, when comparing to healthy controls and chronic pancreatitis (Sarrats *et al*., 2010). However, according to (Matsumato *et al*., 2010), the increase in fucosylated HPT has no significant difference from chronic pancreatitis patients. However, combining them with CA19-9 could improve the diagnostic potential in contrast to the both of the biomarkers isolated. AGP

was also found to be increased in its α-1,3-fucose glycoforms (Giménez *et al*., 2015, Balmaña *et al*., 2016, Mancera-Arteu *et al*., 2017).

α1-Antichymotrypsin (ACT), trombospondin-1 and HPT were the best performing fucosylated glycoproteins in a study by Nie *et al*. (2014) in which lectins (*Aleuria aurantia lectin*) were used analyze the samples. Their combination with CA19-9 proved to have a high diagnostic potential.

ACT also showed a good potential, when analyzed by MS in another study (Tan *et al*., 2015).

Although isolated levels of serum pancreatic RNase 1 are not enough to consider it a potential biomarker, this protein is much more core fucosylated in pancreatic cancer. Quantification of its core fucosylation could therefore be a good biomarker (Barrabés *et al*., 2007). As it was showed that the Asn-88 occupation site is significantly increased in N-glycosylation in pancreatic cancer, this Asn-88 site could also be a novel biomarker (Nakata *et al*., 2014).

Regarding now N-glycan branching of serum glycoproteins, Drabik *et al*. (2017) found four glycoproteins (LIFR, CE350, VP13A and HPT) that carrying an altered N-glycan structure in pancreatic cancer.

Mucins can be secreted from tissues to the bloodstream, making its specific glycoforms easy to detect in serum. Altered glycosylation patterns of mucin (MUC), especially MUC4 (glycoform found in neoplasms: MUC4-Tn) and MUC1 (glycoform found in neoplasms: MUC1-STn) have a good potential as biomarkers (Remmers *et al*., 2013). Immunoprecipitated MUC1 can be studied using lectins (Matsuda *et al*., 2017) or antibody-lectin sandwich arrays with which it was found do carry the CA19-9 antigen (SLe^a) in pancreatic cancer patients (Yue *et al*., 2009). Alterations on MUC1 and CEA were also found by Chen *et al*. (Chen *et al*., 2007) using antibody microarrays.

MUC5AC present in cyst fluid samples can be detected with wheat germ agglutinin lectin and according to Haab *et al*. (2010), can identify malignant pre-cancerous stages.

All of these mucins were described in pancreatic cancer in tissue and not serum (that is the most convenient sample). Due to analytical limitations, O-glycans in mucins cannot be analyzed yet (Llop *et al*., 2018).

Recently reported was also an increased expression of MUC1-SLe^x and MUC5AC-SLe^x (Balmaña *et al*., 2018). MUC5AC-SLe^x is observed in serum from pancreatic cancer patients carrying 3 fucose (Singh *et al*., 2015). Its performance is improved when in comparison with CA19-9 (Tang *et al*., 2016).

Concluding, most of the studies focused on finding altered glycosylation in serum or depleted serum samples. It has mostly resulted in the identification of acute-phase glycoproteins and mucins (MUC1 and MUC5AC). The changes of these, occurred in sialic acid, fucose of $SLe^{x/a}$ occurring mostly in advanced stages of the malignancy or inflammatory stages, limiting its use as early-stage biomarkers. However, combining these aberrant glycolisation with protein levels and imaging could improve its performance (table 14).

Table 14. Glycosylation Biomarkers

Biomarker Panel	Characteristics	Referance
HPT, AGP, CA19-9	Increased core fucosylation	Sarrats *et al*., 2010 Matsumato *et al*., 2010
HPT, ACT, CA19-9	Increased core fucosylation	Nie *et al*., 2014 Tan *et al*., 2015
RNase 1	Increased core fucosylation	Barrabés *et al*., 2007
Asn-88 occupation site	Increased in N-glycosylation	Nakata *et al*., 2014
LIFR, CE350, VP13A and HPT	Altered N-glycan branching	Drabik *et al*., 2017
MUC4 (glycoform found in neoplasms: MUC4-Tn)	Altered glycosylation patterns	Remmers *et al*., 2013
MUC1 (glycoform found in neoplasms: MUC1-STn)	Altered glycosylation patterns	Remmers *et al*., 2013 Chen *et al*., 2007
MUC5AC	Altered glycosylation patterns	Haab *et al*., 2010
MUC1-SLe^x	Increased expression	Balmaña *et al*., 2018
MUC5AC-SLe^x	Increased expression, carrying 3'fucose	Balmaña *et al*., 2018 Singh *et al*., 2015
MUC1 and MUC5AC, protein levels and imaging	Changes in sialic acid, fucose of SLe$^{x/a}$ in advanced stages hence the combination	Llop *et al*., 2018

3.3 Lectins as a tool

Lectins are ubiquitous proteins. They show a specificity for the carbohydrate moiety of glycoconjugates (Hashim *et al*., 2017) being defined as glycan-specific receptors. They are the so-called decoding system for the oligosaccharide codes (Ribeiro *et al*., 2018). It has been difficult to classify lectins, due to their immense diversity. However, nowadays lectins are classified according to distinct protein folding, domains/structural similarities and evolutionary-relatedness of proteins (Peumans *et al*., 2001). According to these criteria, there are twelve lectin families, (Van Damme *et al*., 2008). The first lectin to be discovered is believed to have been ricin in 1888 (Sharon *et al*., 2004). It

is an extremely toxic protein and can be found in *Ricinus communis*. Like ricin, most of lectins have a very high cytotoxicity. Since then, there are more than a thousand plants reported to possess lectins and it is believed that a large number still remains to be discovered (Ribeiro *et al*., 2018). Due to their carbohydrate binding specificities, lectins have been thoroughly studied, having innumerous applications now-a-days. Lectins can be found in plants (mostly as seed storage proteins (Ribeiro *et al*., 2014)) and fungi and very dispersed in nature.

3.3.1 Biomedical Application

In biomedical research, lectins play an important role. Whether in cancer, as a means of discovering new biomarkers, whether in neurodegenerative diseases or as a means of target-therapy (Ribeiro *et al*., 2018).

Due to the specificity of lectins, they can recognize carbohydrates on the cellular membrane, making them excellent tools for decoding the abnormal glycosylation happening in malignancies. Many of these aberrantly glycosylated expressed peptides in cancer patients, are not possibly detected using conventional methods due to the fact that they occur in very low concentrations. In combination with mass spectrometry, lectins can help identifying several potential cancer biomarkers, based on glycosylation degree (Mody *et al*., 1995). Most of the methods in which lectins are used to identify aberrations in glycolysation in malignancies consist on adaptations of other methods normally used with antibodies (Enzyme-linked lectin assay, lectin histochemistry, lectin blotting).

Furthermore, it has also been found that there are some lectins with anticancer properties. As a result of lectins dictating cytotoxic effects and mediating apoptosis (Park *et al*., 2000) and autophagy they can prevent tumor growth.

Programmed cell death can also be one effect caused by lectins, when used as anticancer therapy (Ribeiro *et al*., 2018).

Lectins also have great potential as tissue specific therapy due to its specificity for a specific oligosaccharide side chain (Ribeiro *et al*., 2018).

3.3.2 Immobilized-Lectin Affinity Chromatography

Immobilized-lectin affinity chromatography consists on immobilizing lectins onto a matrix and its carbohydrate ligands (figure 12). It is a method making use of the specificity of lectins interactions aiming for glycoproteins separation and enrichment (Hage *et al*., 2012). As a technique used for identifying potential biomarkers, it is

normally used in addition with mass spectrometry analysis. By running a sample, the result is a glycoprotein-enriched eluate. Comparing, a healthy control and a pancreatic cancer patient samples, there can easily be identified the aberrantly expressed or glycosylated glycoproteins. For pancreatic cancer, there has been identified a potential biomarker with this technique. The enrichment of core fucosylated glycoproteins was carried out using *Lens culinaris* agglutinin in combination with mass spectrometry analysis (Tan *et al*., 2015).

Another used variant is the use of a panel of lectins, instead of just one. Making use of the complement of the panel multi-lectin affinity chromatography gives a deeper analysis (Hashim *et al*., 2017).

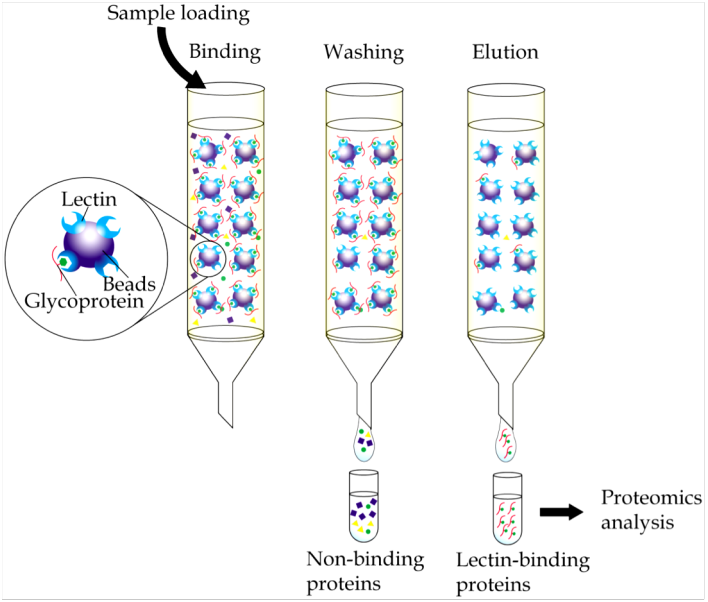


Figure 12. Immobilized-lectin affinity chromatography

(adapted from Hashim *et al*., 2017)

3.3.3 Enzyme-linked Lectin Assay

Enzyme-linked lectin assay consists in the replacement of antibodies by lectins in the enzyme-linked immunosorbent assay (Hashim *et al*., 2017). The principle adopted remains the same. The enzyme-linked immunosorbent assay (McCoy *et al*., 1983) is a direct assay in which to the sample in a microtiter plate is added an enzyme-conjugated lectin. While the lectin specifically binds to the glycan moiety, the enzyme converts the substrate into a colored product. The intensity of the coloration is measured with the help of a spectrophotometer and used to estimate the glycoconjugates level. This method is widely used, including in the research of cancer

biomarkers (Kuzmanov *et al*., 2013), as it is quick, easy and requires only small amounts of samples. However, the correct identification of glycoproteins may only be possible by coupling enzyme-linked lectin assay with proteomics analysis or antibody detection.

For this method, there are three different and possible approaches (figure 13): (a) The direct assay, the classical protocol, as already told consists on the addition of an enzyme-conjugated lectin to the microtiter plate containing the sample. (b) In the hybrid assay it is the other way around, the plate contains the antibody capturing the specific glycoproteins, only after this is added the enzyme-conjugated lectin. (c) Sandwich enzyme-linked lectin assay involves two lectins and is almost a combination of the first to methods. One lectin is on the plate and captures de glycoprotein. The second lectin is used as a detection reagent.

Ching *et al*. (1989) identified a glycoprotein in the serum of pancreatic cancer patients that binds to peanut lectin. With the purpose of identifying it and diagnosing patients, Ching *et al*. (1989) developed a direct enzyme-linked PNA assay. The results (in terms of sensibility and specificity) were similar to the ones using CA19-9.

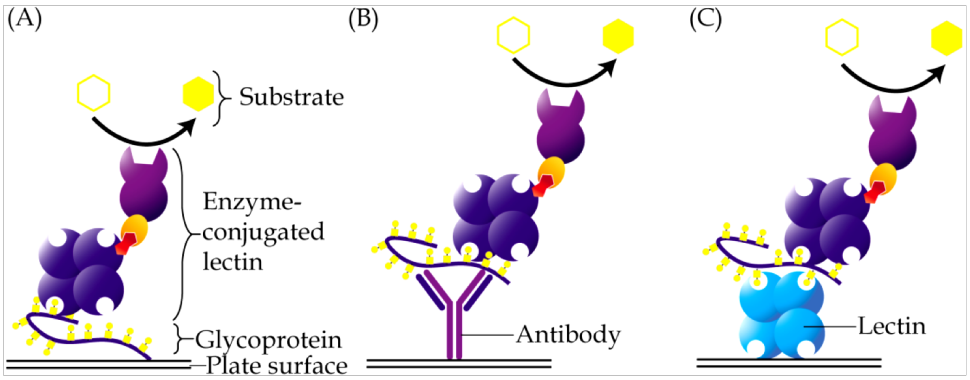


Figure 13. Enzyme-linked lectin assay approaches

(adapted from Hashim *et al*., 2017)

3.3.4 Lectin Histochemistry

Lectin histochemistry is very similar to immunohistochemistry. It too consists in visualizing cellular components of tissue microscopically, but the antibodies are replaced by lectins (Hashim *et al*., 2017). It can be used to obtain information about the glycosylation present in the sample. Comparing healthy controls with cancer samples may highlight aberrant glycosylations. There are two variants possible (figure 14): (a) the direct method, in which a lectin is covalently bonded to fluorophores, enzymes, colloidal gold or ferritin. (b) in the indirect method, a hapten (alone cannot

induce an immune response but when linked to a higher molecular weight carrier protein generates one), namely a bioten or digoxigenin is conjugated with a lectin, they are then recognized by an enzyme linked-streptavidin (highly selective to bioten making one of the strongest links) or -anti-digoxigenin (Roth *et al*., 2011).
Lectin histochemistry has already been widely used for studying glycolysation aberrations in malignancies (Sobral *et al*., 2010).

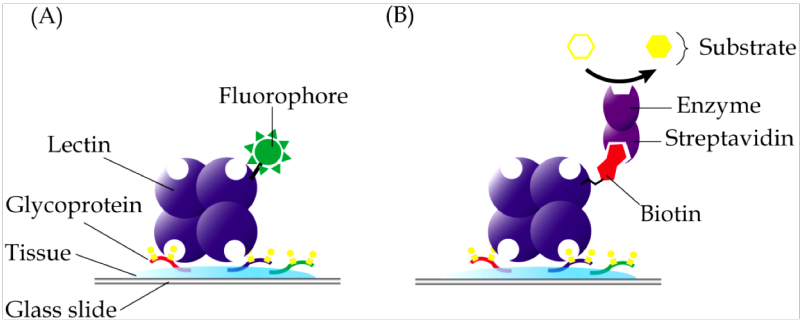


Figure 14. Lectin histochemistry
(adapted from Hashim *et al*., 2017)

3.3.5 Lectin Blotting

As a result of replacing the antibodies used in western blotting by lectins arises the lectin blotting (Shan *et al*., 2001). As in the western blot, samples are placed on a polyacrylamide gel and transferred (using current) onto a polyvinylidene fluoride or nitrocellulose membrane (figure 15). Glycoproteins are then detected by lectins and the visualization of the complex is possible by the use of conjugates (enzymes, biotin, digoxigenin, …), like in histochemistry. Lectin blotting has been used for characterizing glycan structures (Akama *et al*., 2006), detecting and quantifying N- or O-glycosylated proteins (Roth *et al*., 2012) and detecting aberrations in glycosylation (like it happens in cancer) (Kitamura *et al*., 2003). However, it does not have very potential in routine diagnostics (Hashim *et al*., 2017).

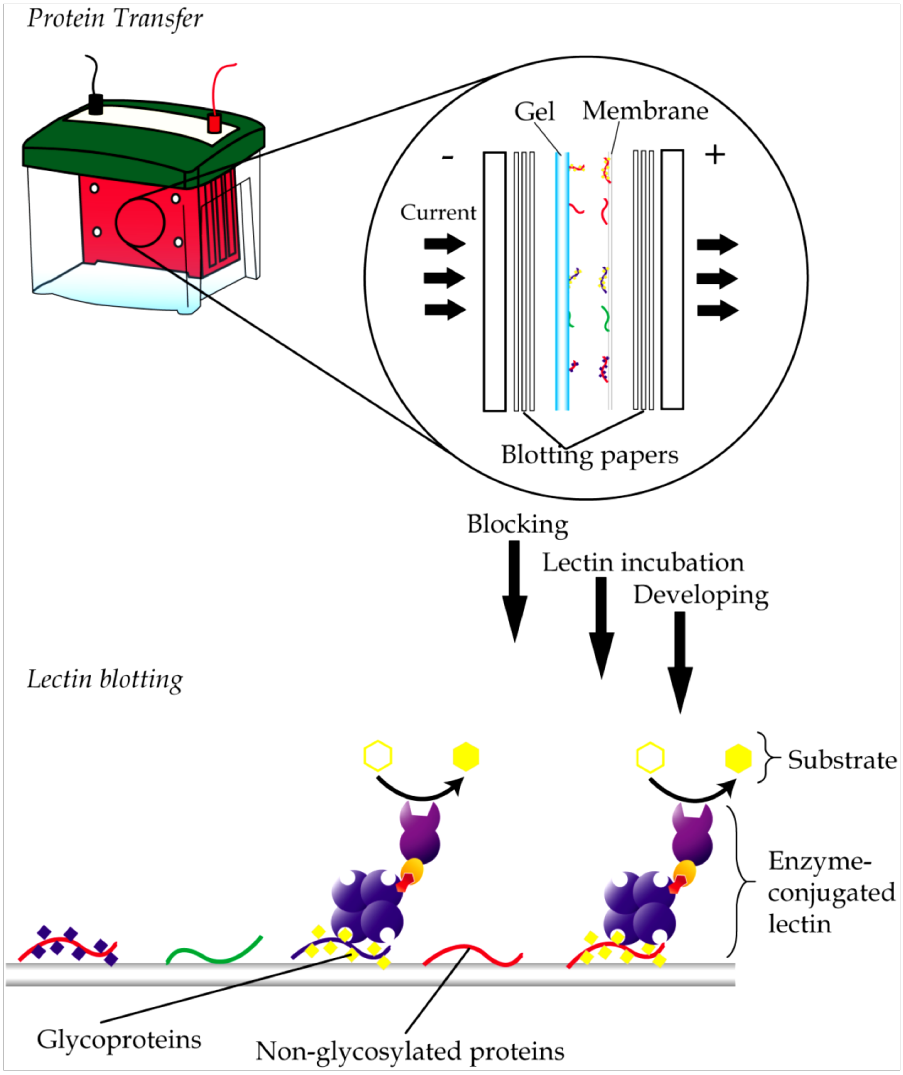


Figure 15. Lectin blotting

(adapted from Hashim *et al*., 2017)

3.3.6 Lectin Array

On the lectin array, multiple lectins are immobilized onto a solid support. Due to the fact that there are different lectins, with different specificities, different glycoproteins can be detected at the same time (Hu *et al*., 2009, Hirabayashi *et al*., 2011). It is a quick and sensitive analysis of glycans (Hashim *et al*., 2017). Multiple potential biomarkers have already been identified with the help of lectin array, namely *Agaricus bisporus* lectin for colorectal cancer (Nakajima *et al*., 2015). Lasectin array has been modified several times already. To this point, there are two variants being used (figure16): (a) one lectin per spot are organized into the lectin array slide. When the samples come in contact with the lectins, the spots where the specific glycoproteins are illuminated under appropriate scanner due to their interactions. (b) another

possibility is to conjugate lectins to different fluorescent colored beads. After entering in contact with samples, the beads pass through a detector with two lasers, one identifies the beads (classification laser) and the other quantifies them (reporter laser) (Hashim *et al*., 2017).

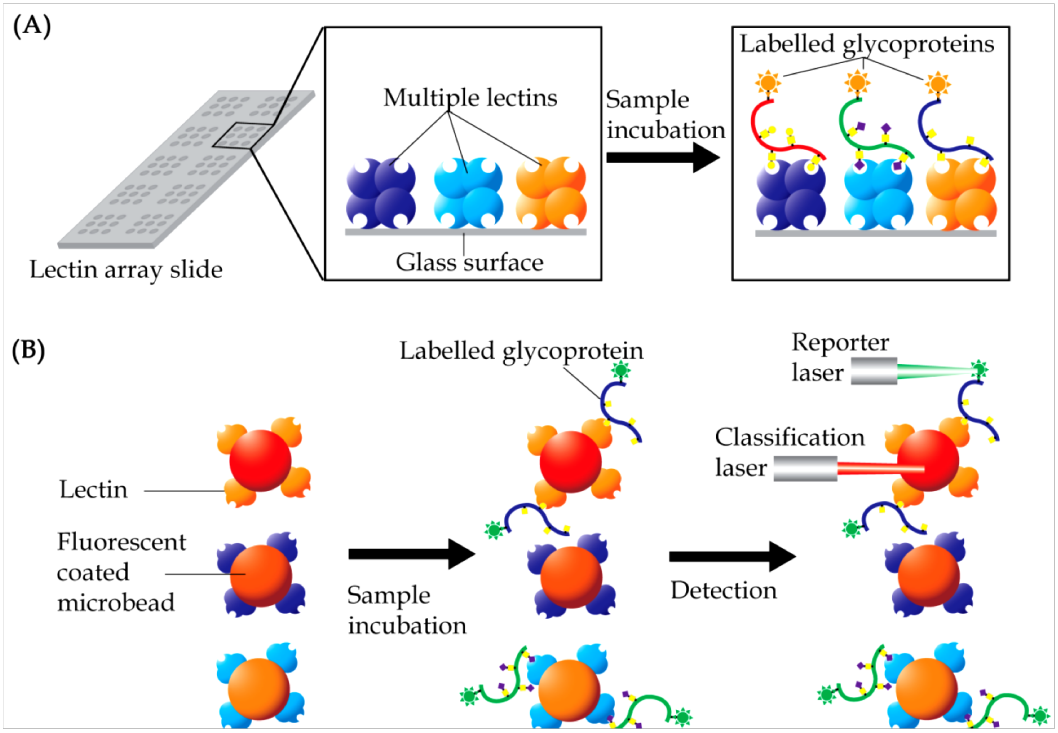


Figure 16. Lectin array

(adapted from Hashim *et al*., 2017)

4 Conclusions

Pancreatic cancer remains an extremely challenging disease. It is asymptomatic, lacks radiological manifestations and there still is no known specific molecule circulating in body fluids. It is only curable in early stages, showing no symptoms by this time.

To distinguish the malignancy in early-stage with adequate sensitivity and specificity, screening first with biomarkers and then imaging could be the solution. Development of a screening protocol for high-risk populations is crucial to enhance life expectancy. However, there are innumerous possible biomarkers.

As problems in development of biomarkers there is the fact that most patients are diagnosed in advanced stages. Due to this, having samples from early-stage available for screening for biomarkers is even more difficult. A possible way to overcome this,

could be to screen high-risk populations with positive family history. Controlling their biomarker levels once a year could be a possibility to detect a significant and specific increase (or decrease) in biomarkers, which could be used in clinical practice as early-stage biomarkers.

To evaluate its validity, these studies also need controls, specifically samples from benign pancreatic diseases. This could also not be easy to find.

The solution seems to come from developing a panel with CA19-9 and other biomarkers in order to improve sensitivity and specificity. CA19-9 is the only biomarker used in pancreatic cancer (for monitoring patients). Its main problem as an early-stage biomarker is due to the fact that it is not expressed in 10-20% of the Lewis antigen-negative population. Combining it in a panel with other biomarkers is promising. However, there still is a lot of work to be done in furtherly studying and validating the many different existing possibilities.

The most promising combinations can be seen table 15.

Table 15. Best performing potential biomarkers

Biomarker Panel	CA 19-9, albumin and IGF	CA 19-9, albumin, C-reactive protein and interleukin	CA19-9, TIMP1, LRG1	CA19-9, *THBS2*	Palmitic acid	Ezrin	CA19-9, miR-145, miR-150, miR-223, miR-636	CA19-9, miR-26b, miR-34a, miR-122, miR- 126, miR-145, miR-150, miR-223, miR-505, miR-636, miR-885.5p	CD44v6, Tspan8, epcam, MET, CD104, miR-1246, miR- 4644, miR-3976, miR-4306
Results	Sensitivity: 93,6%; Specificity: 95%	Sensitivity: 99.39%; Specificity: 90%	AUC: 0.95; Sensitivity:75%; Specificity: 95%	Sensitivity:87%; Specificity: 98%	AUC: 1.0; Sensitivity: 100%; Specificity: 100%	AUC: 0.9 Sensitivity: 93.2% Specificity: 75.5%	AUC: 0.93; Sensibility: 85%; Specificity: 85%	AUC: 0.93; Sensitivity: 85%; Specificity: 85%	Sensitivity: 100% Specificity: 80%
Referance	Goh *et al.*, 2017	Zeh *et al.*, 2005	Capello *et al.*, 2017	Kim *et al.*, 2017	Di Gangi *et al.*, 2016	Capello *et al.*, 2013	Schultz *et al.*, 2014	Schultz *et al.*, 2014	Madhaven *et al.*, 2015

5 Challenges and Future Directions

Finding an early-detection biomarker for pancreatic cancer passes by finding a combination of biomarkers that complement each other in order to achieve the best sensitivity and specificity. When using it in combination with imaging techniques, it should improve its results. Large-scale studies using the same standardized methods and conditions are needed to validate the results already obtained.

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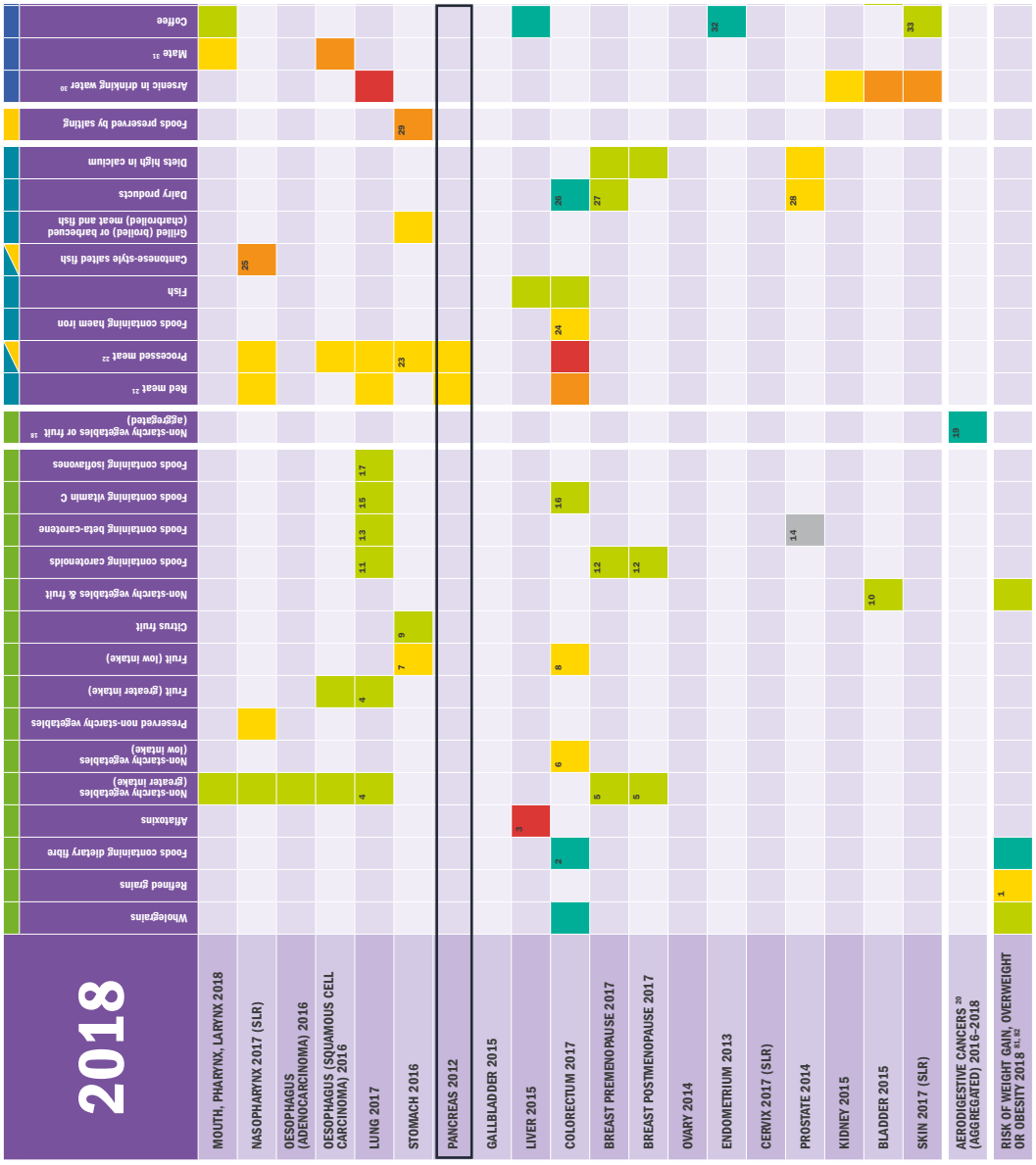
Annexes

A1. The evidence for cancer risk: summary matrix



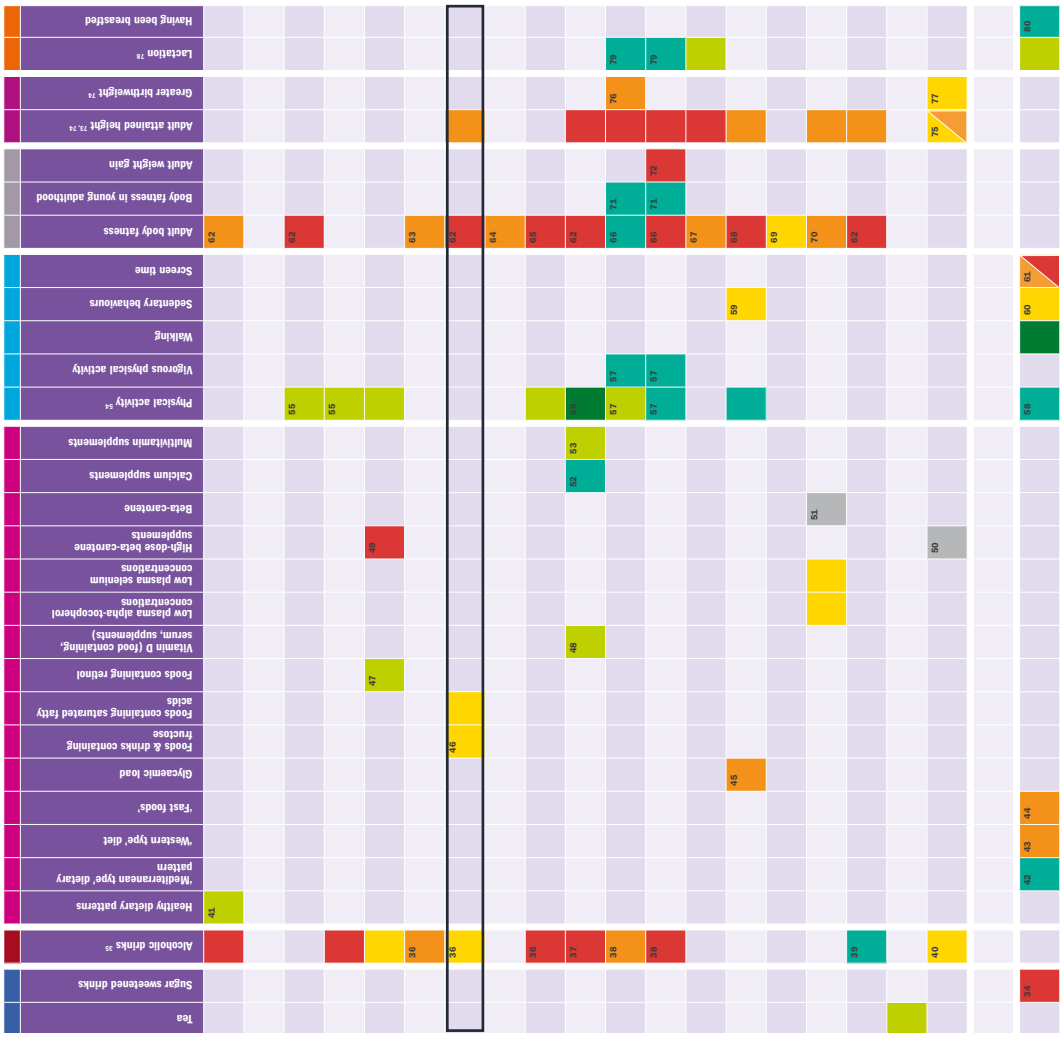
Part 1. Adapted from World Research Fund, 2018

Conclusions Key

- Convincing decreases risk
- Probable decreases risk
- Limited – suggestive decreases risk
- Convincing increases risk
- Probable increases risk
- Limited – suggestive increases risk
- Substantial effect on risk unlikely

Exposure Group Key

- Wholegrains, vegetables and fruit
- Meat, fish and dairy products
- Preservation and processing of foods
- Non-alcoholic drinks
- Alcoholic drinks
- Other dietary exposures
- Physical activity
- Body fatness and weight gain
- Height and birthweight
- Lactation/having been breastfed



Part 2. Adapted from World Cancer Research Fund, 2018

Conclusions Key

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